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Transport of phage P22 DNA into the cytoplasm of *Salmonella enterica* serovar Typhimurium

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

in

Biology

by

Gerardo Legrama Perez

Committee in charge:

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Professor Constantine Tsoukas

2008

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2008

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ABSTRACT OF THE DISSERTATION

Transport of phage P22 DNA into the cytoplasm of *Salmonella enterica* serovar Typhimurium

by

Gerardo Legrama Perez

Doctor of Philosophy in Biology

University of California, San Diego, 2008
San Diego State University, 2008

Professor Stanley R. Maloy, Chair

The short noncontractile tail of P22 allows it to eject its DNA together with three phage-encoded proteins (gp7, gp16, and gp20) into the periplasm of the host. However, the mechanism of phage P22 DNA transport across the cytoplasmic membrane of *Salmonella enterica* sv. Typhimurium is not known. This process could be mediated by phage-encoded or host-encoded proteins, or a combination of both. Genetic and biochemical approaches failed to identify host factors that are essential for P22 DNA uptake, suggesting that phage-encoded proteins are sufficient to catalyze P22 DNA uptake. This hypothesis was tested by studying the protein-protein interactions, the DNA-binding properties and the membrane partitioning

characteristics of the P22 ejected proteins. The energy requirements for P22 DNA transport was also tested by studying the effects of inhibitors of the ATP synthase and uncouplers of the proton motive force. The pull-down assays revealed that the P22 ejected proteins interacted with each other. The gel retardation assays showed that these proteins not only bound DNA nonspecifically but also protected the DNA from degradation by DNase I. The protein gp16 partitioned into the detergent phase of Triton X-114 and into the liposomal fraction. Gp16 was also found to disrupt membranes by possibly forming a channel across the liposomal membrane. When ^{32}P-labeled DNA was used to detect the transport of DNA inside liposomes, transport of radiolabeled DNA into liposomes was possible only in the presence of gp16 and an artificially-created membrane potential. The requirement for the membrane potential in the transport of phage P22 DNA into the cytoplasm of *Salmonella* was shown when valinomycin plus potassium chloride reduced the transduction efficiency of the host cells by 63%. The findings reported in this thesis suggest that the transport of phage P22 DNA across the cytoplasmic membrane of the *Salmonella* host is mediated by the phage-encoded protein gp16, and that transport is driven by the membrane potential of the host. The primary role of the other ejected proteins, gp7 and gp20, might be to protect the ejected DNA from periplasmic nucleases.

Chapter 1. Phages and their specific hosts

Bacteriophages (also known as phages) are ubiquitous in our world. They are found in the oceans, soil, deep sea vents, the water that we drink, and the food that we eat (94). They are the most abundant living entities on the planet – the estimates range from 10^{30} to 10^{32} in total – and play key roles in regulating the microbial balance in every ecosystem where this has been explored (19). Metagenomic analyses have shown that environmental viral communities are incredibly diverse. There are an estimated 5000 viral genotypes in 200 liters of seawater and possibly a million different viral genotypes in one kilogram of marine sediment (19).

Phages played a critical role in the development of molecular biology. The results of phage research were very important in making some of the most significant discoveries in the history of the biological sciences, including the identification of DNA as the genetic material, the discovery of the transduction phenomenon, the deciphering of the genetic code, and the discovery of the messenger RNA (94, 171).

Significance

In addition to their value as biological models, there are practical reasons for studying phages. One reason is that phage often carry virulence factors that enhance bacterial pathogenesis (13). In many cases, a bacterial host is avirulent until infected with a phage that encodes an exotoxin (Table 1.1). An example of this is the CTX phage which carries the gene that encodes for the cholerae toxin. Upon infection of the *Vibrio cholerae* host, the CTX phage integrates into the bacterial chromosome, allowing V*ibrio cholera* to express the cholera toxin and potentially cause disease in humans. STX, clostridial phages, corynephage β, and T12 are other examples of

toxin-carrying phages that could allow their specific host to express the toxins upon phage infection and eventually cause harm in humans. In this specific interaction between the phage and the bacterial host, preventing the infection of the bacterial host by the phage would minimize bacterial pathogenesis.

Table 1.1: Infection of specific bacterial host by exotoxin-carrying phages.

Bacterium	Phage	Gene Product	Disease	References
Vibrio cholerae	CTX phage	cholerae toxin	Cholera	(13, 33)
Escherichia coli	STX phage	shigalike toxin	Hemorrhagic diarrhea	(13, 180)
Clostridium botulinum	clostridial phages	botulinum toxin	Botulism (food poisoning)	(13, 73)
Corynebacterium diphtheriae	corynephage β	diphtheria toxin	Diphtheria	(13, 73)
Streptococcus pyogenes	T12	erythrogenic toxins	Scarlet fever	(6, 13)

Another compelling reason why studying phages is important is because phage may be enlisted as antibacterial agents (107, 113). Phages can be used in combination with antibiotics to cure and prevent the spread of bacterial infections that are caused by antibiotic-resistant pathogenic bacteria (Table 1.2). An example of this is phage K which was found to eradicate methicillin-resistant *Staphylococcus aureus* infections or MRSA. Another example is the concoction of 5 different phages that are specific in killing *Listeria monocytogenes* which is a bacterium that can survive in the refrigerator. This anti-*Lysteria* concoction called LMP-102 was approved by the FDA 2 years ago and is now being used as an additive in meat and poultry products to

prevent the growth of *Listeria*. Lysin is a protein extracted from a phage that can be used to kill *Bacillus anthracis* which is the bacterium that causes anthrax. In applications where phage are used for antibacterial purposes, increasing the efficiency of phage infection would enhance the killing of pathogenic bacteria.

Table 1.2: Phages or phage-derived proteins that have been shown to eradicate bacterial infection by infecting and killing their specific host cell.

Bacteria	Phage or phage protein	Assay	References
Staphylococcus aureus	MR11	Mice	(114)
Methicillin-resistant *Staphylococcus aureus* (MRSA)	K	*In vitro*	(125, 126)
Pseudomonas aeruginosa	KPP10	Mice	(184)
Vancomycin-resistant *Enterococcus faecium*	ENB6	Mice	(14)
Listeria monocytogenes	LMP-102	Meat & poultry	(98)
Bacillus anthracis	Lysin	*In vitro*	(129)

Specific Aims

Understanding how phage DNA is translocated into the host cytoplasm is important for two reasons. Many phages carry virulence factors that can make their

host more pathogenic and infection of humans with this pathogenic strain of bacteria can cause a deadly disease. If the phage DNA can be prevented from entering the host cytoplasm, then the pathogenicity of the host bacterium can be minimized. In another scenario wherein the bacterial host is resistant to a number of antibiotics, it would be beneficial to humans if we could lyse these antibiotic-resistant bacteria by increasing the efficiency of phage infection. To determine how phage DNA is translocated across the cytoplasmic membrane of the bacterial host, the interaction between phage P22 and its specific host *Salmonella enterica* sv. Typhimurium was investigated. The aims of this project were to:

I. Determine the bioenergetic requirements for the translocation of phage P22 DNA across the cytoplasmic membrane

II. Determine whether the P22-encoded pilot proteins (also known as ejected proteins) are capable of forming a channel that allows transport of the phage DNA across the cytoplasmic membrane

III. Determine whether host-encoded factors are required for translocation of P22 DNA across the cytoplasmic membrane

Background

Transport of highly hydrophilic DNA molecules across the hydrophobic membrane barrier may be analogous to the process of importing proteins into the cell. Protein export is essential in prokaryotic cells and the mechanism has been dissected in detail (103, 116, 140, 152). Several distinct mechanisms mediate DNA transport into bacteria, including natural transformation, conjugation, and uptake of DNA from

phage. Natural transformation and conjugation have been thoroughly studied in several systems (37, 109, 138, 139). In the DNA transport systems that are best understood, a single strand of DNA is brought into the bacterial recipient. In contrast, uptake of double stranded phage DNA remains poorly understood.

DNA ejection in tailed phages

The order of tailed phages, *Caudovirales*, contains three families: *Myoviridae*, *Siphoviridae,* and *Podoviridae* (149). Phages belonging to these three families have contractile, long noncontractile or short noncontractile tails, respectively. Among the best studied tailed phages are T4 in the *Myoviridae* family, λ in the *Siphoviridae* family, and T7 and P22 in the *Podoviridae* family. Although each of these phages has been extensively studied, many questions remain about how the phage DNA enters the host cell. The process of DNA uptake can be divided into three steps: (1) Penetration of the outer membrane, (2) Ejection of phage DNA and associated proteins and (3) Transport of the DNA into the cytoplasm.

I. Uptake of phage T4 DNA

Phage T4. Like most phage, T4 has a genome consisting of a single molecule of nucleic acid. The approximately 172 Kbp linear duplex DNA of T4 is one of the largest known phage genomes (57). The T4 genome is encapsidated within a protective head. Attached to the head, T4 has a tail which serves as an adsorption organ and a channel for transfer of DNA into the infected cell. T4 is only capable of undergoing a lytic life cycle. The lytic lifecycle (from entering a bacterium to its destruction) takes approximately 30 minutes (at 37 ℃) and consists of: (1) adsorption and penetration,

(2) arrest of host gene expression, (3) enzyme synthesis, (4) DNA replication, and (5) formation of new phage particles. After the lifecycle is complete the host cell bursts open and releases the phage progenies into the environment. Burst sizes or phage yields are relatively large, usually 200 phage particles or more per cell.

T4 biology is distinctive in several ways (57). The genome is circularly permuted with respect to nucleotide sequence, and about 3 Kbp of DNA is repeated at both ends of the packaged DNA. The DNA nucleotides are modified by covalently-linked glucose moieties on Hm-dCMP hydroxymethyl groups, modifications that protect the DNA against host restriction systems. The numbers of viral substructures and structural proteins (nearly 50) exceed those found even in most other large-tailed phages. T4 uses RNA polymerase of the host cell for transcription of all its genes (57).

Reversible attachment. The interaction between T4 and *E. coli* is initiated when phage tail fibers recognize certain components in the outer membrane and mediate attachment of the phage. Wilson *et al*. (186) showed that T4 tail fibers bind specifically to purified *E. coli* lipopolysaccharide (LPS) and Jesaitis and Goebel (72) identified LPS as the specific receptor for T4 adsorption by using phenol extracts of *E. coli*. In addition, Dawes (34) demonstrated that T4 adsorption is blocked by specific mono- and disaccharides. Based upon these studies, it is now known that T4 tail fibers attach specifically to the glucosyl-α-1, 3-glucose terminus of rough LPS in *E. coli* B or to OmpC (37 kDa) in *E. coli* K (34, 132). When the phage initially recognizes the host via reversible interaction of the tips of its long tail fibers with lipopolysaccharide cell surface receptors, the baseplate is about 100 nm away from the cell surface (104).

The six long tail fibers are symmetrically connected to the hexagonal baseplate. If one tail fiber interacts with an LPS molecule, it is more likely that others will follow suit. Moreover, if a bound tail fiber becomes unbound, it is likely to bind again nearby, since the LPS concentration on the cell surface is high (57). There are about 10^6 LPS monomers on a cell surface of about 2 X 10^6 nm^2, based on a cylindrical bacterium 1 um long and 0.5 um in diameter (166). The LPS molecules are distributed on the cell surface in tufts of three. Binding of the phage to the cell surface is reversible if the next stage of baseplate interaction does not occur. Reversibility of adsorption suggests that the individual tail fiber-LPS interaction is weak. Wilson *et al*. (186) demonstrated that only about 30% of isolated tail fibers bind specifically to excess isolated LPS. Weak binding of the tail fibers to a dense LPS lawn on the cell surface would allow the phage to wander across the two-dimensional surface as the tail fibers detach and attach again at random, but would tend to restrict them from rising off the cell surface into the medium (57). When a minimum of three long tail fibers have bound to the host cell receptors, the fibers change their conformation, thereby signaling to the baseplate that binding has been successful (149). Subsequently, the long tail fibers are used as levers to move the baseplate toward the cell surface (104).

Irreversible attachment. While reversible adsorption of T4 involves the tail fibers, irreversible adsorption occurs through the baseplate. Certain baseplate proteins (gp9, gp11, and gp12) play key roles in irreversible adsorption (57). Gp9 attaches tail fibers to the baseplate (31) while gp12 makes a fibrous trimer which is attached to gp11 on the baseplate during morphogenesis (32, 89-91). Morphological evidence showed by Simon and Anderson (165) implicated the baseplate gp12 fibrils

as the organelles that irreversibly attach the phage to the cell surface. In thin sections of phage-infected bacteria, the gp12 fibrils are seen connecting the baseplate to the cell surface.

Recognition of an appropriate area of the host surface by the tail fibers signals other tail structures so that the baseplate binds irreversibly, thereby leading to DNA ejection (57). The attached tail fibers signal the phage by changing its angle to the baseplate as the latter approaches the cell surface. The cooperative descent of the phage tail fibers to the cell surface destabilizes and triggers the release of the baseplate pins that secure the hexagonal conformation of the baseplate, causing the irreversible expansion of the baseplate from a hexagon to the wider hexagram (star) form. The six gp12 trimers transform to form long, thin fibrils permanently joining the outer baseplate wedges to the cell surface (104, 149). When the baseplate irreversibly expands from the hexagon to the star form, an opening is made in the center of the baseplate through which the tail tube can extrude. The tail sheath (gp18) contracts to expose the lower half of the tail tube (gp19), bringing the baseplate closer to the phage head and farther from the cell surface. Calorimetric measurements have demonstrated that sheath contraction releases approximately 6000 kcal per mol of tails (4). This energy is used to create an opening in the outer cell membrane so that the tail tube can be inserted into the periplasmic space (149). The rigid tail tube is driven through the outer cell membrane by the “membrane-puncturing device” that is formed by the gp5 C-terminal β-helix, situated at the tip of the tube extension. The β-helix dissociates when it comes in contact with the periplasmic peptidoglycan layer, thus activating the three lysozyme domains of gp5. The lysozyme digests the peptidoglycan layer to create an opening through which the

tail tube can reach the cytoplasmic membrane of the host cell. The contact of the tail tube with the cytoplasmic membrane initiates release of the phage DNA into the host through the tail tube (149).

DNA ejection. Early electron micrographs (49, 122) led to a common misconception that adsorbed phage contract like a hypodermic syringe and thereby inject their DNA into the bacterial cytoplasm. This implies that the release of pressure in the phage head forces the DNA across the membrane for which there is no evidence (57). It is now clear that the contraction of the tail sheath is not a musclelike phenomenon (i.e. a reversible alteration in tail sheath length similar to actin and myosin), but rather the relaxation from a high-energy to a preferred low-energy state (104). The tail sheath goes from a long, narrow cylinder covering the inner tail tube to a wider and shorter cylinder only partially covering the tail tube. This contraction of the sheath is triggered by expansion of the baseplate at the distal end (120). Tail sheath contraction and DNA ejection are not linked since tail contraction induced by concentrated lipopolysaccharide or 3 M urea (30) does not cause the release of phage DNA (57, 104). Phages with contracted tails can infect spheroplasts (bacteria stripped of the outer membrane) of *E. coli*, suggesting that the release of the phage DNA can be triggered only by interaction of the tail tube with the *E. coli* cytoplasmic membrane (57, 104). DNA is probably not injected directly into the cytoplasm, since the tail tube cap seems to only reach the outer surface of the cytoplasmic membrane after tail sheath contraction (164). Thus, the terms DNA “ejection” and “uptake” have become more common in describing the exit of DNA from the phage particle and the entry of DNA into the cell cytoplasm, respectively.

At present, the mechanism of T4 DNA ejection is poorly understood. Technical problems have made examination of the mechanism of T4 DNA ejection particularly difficult. DNA transfer is accomplished in less than 1 min (>3000 bp/s) under optimal conditions. The high rate and efficiency of transfer suggest that there is a vectorial engine directing the DNA into the cell (57).

Little is known about the constituents that may participate in the recognition, formation, and closing of channels for DNA uptake. There are, as yet, no data to show which host and/or phage proteins are involved in the transfer of phage T4 DNA. It has been postulated that contact of the cap with a specific site on the membrane surface is normally required to trigger phage DNA release (7, 48). If so, the cell surface site combines with the tail tube, and together they form a channel for DNA passage into the cytoplasm, thus protecting the entering DNA from periplasmic nucleases. The efficiency of infection may well depend on the ability to form this protective channel. The ability of many species of spheroplasted enteric bacteria such as *Serratia*, *Proteus*, *Salmonella*, and *Aerobacter* spp. (183) to host productive infection by contracted T4 particles implies either that the site of the interaction with the tail tube cap is rather nonspecific or that it has some common protein that has not diverged much since the time that the outer surfaces of enteric bacteria acquired their structure (57).

Bayer (8, 9) reported adhesion zones between inner and outer membranes at sites of T4 phage attachment. Electron micrographs suggested that the transfer of macromolecules usually occurred at such sites. It is still debatable whether contact sites represent native stable structures, which contain the specific sites of potential phage attachment, or if they are stabilized upon infection by phage or translocation of

macromolecules (48, 61). On the basis of freeze-fracture electron microscopy and thin-sectioning observations, Tarahovsky *et al*. (177), proposed that infection by phage T4 results in the formation of fusion sites between the two membranes.

To initiate DNA uptake, the membrane potential, but not the pH gradient, is required (95). If the pH gradient is collapsed while leaving the membrane potential intact, DNA uptake is normal. When the membrane potential is regulated by adjusting the concentration of K^+ in the presence of valinomycin, there is a threshold (ca. 60 to 90 mV) below which DNA no longer enters the cytoplasm and above which DNA enters normally (96). Lettelier *et al*. (105) previously concluded that T4 DNA transport takes place through a phage protein forming a voltage-gated channel that opened above a threshold of membrane potential and remained open only during DNA transport. They speculated that the membrane potential dictates the conformation of gp5 and its insertion into the cytoplasmic membrane. They added that it is unlikely that the DNA is mechanically pulled by the host polymerase since transport takes place within 30 sec at a rate of about 4000 bp per second (see section on phage T7 DNA uptake into *E. coli* for comparison of rates of DNA uptake).

II. Infection of *Escherichia coli* by phage λ

Phage λ. λ contains a double stranded DNA that is kept in the phage head by a long noncontractile tail (18, 137). λ has been studied extensively, and the complete sequence of the λ genome has been determined (155). λ infects most laboratory strains of *E. coli*. However, many natural isolates including pathogenic strains of *E. coli* are resistant to λ because they are encapsulated or produce a fully polymerized O-antigen that interferes with adsorption (101).

Penetration of the outer membrane. Steps leading to the penetration of the

outer membrane are the adsorption of the phage to a receptor on the outer membrane and ejection of the DNA from the phage head. The λ receptor was identified by isolation of *E. coli* mutants that were resistant to λ (λ^R) (101). Approximately 80% of the isolated λ^R mutants were unable to use maltose as a carbon source (Mal^-) (102). These λ^R Mal^- mutations fell into three classes (178). (i) Mutations in the *malT* or *crp* genes that prevent expression of the *mal* operon. (ii) Polar mutations in the *mal* operon that prevent expression of the *lamB* gene. (iii) Mutations in the *lamB* gene which abolish binding of both maltose and λ. Much rarer λ^R Mal^+ mutants were due to mutations in the *lamB* gene that disrupt the λ-binding domain but do not interfere with the maltose-binding domain of the LamB protein.

Host range mutants of phage λ (called λ *h*) were isolated that were able to infect mutants of *E. coli* resistant to wild-type λ (3). The altered host range of the λ *h* mutants was due to a mutation in the λ *J* gene which encodes for the phage tail fiber (163). Substituting the *J* gene from λ with the tail fiber gene from the closely related phage 434 resulted in a hybrid phage with the host specificity of phage 434 (47), which uses OmpC porin as a receptor (160). These studies strongly suggest that the λ *J* gene product (gpJ) determines the host specificity of the phage (101).

Genetic evidence indicates that gpJ directly interacts with the LamB protein in the *E. coli* outer membrane (101). Some *lamB* missense mutations result in resistance to wild-type λ, but remain sensitive to λ *h* mutants (178). Null mutations in the *lamB* gene result in resistance to both λ and λ *h* phages, indicating that the phage host range mutants do not use a different outer membrane receptor (178).

Phage λ binds to unilamellar liposomes bearing the LamB protein (145, 147), and the λ DNA can be ejected into the internal aqueous space (144). Roessner and

Ihler (144) reported the existence of stable transmembrane channels formed in association with DNA ejection into liposomes and their absence from various complexes which do not result in DNA ejection. The molecular diameter of the transmembrane channel is smaller than 5 nm since it permitted entry and escape of small molecules, but not proteins. The nature of the transmembrane channels formed in association with DNA ejection remains unknown. Roessner and Ihler (144) speculate that the channel may be composed of the λ tail protein (gpJ), LamB, or both.

Uptake of DNA across the cytoplasmic membrane. In addition to mutations in outer membrane components, some λ^R mutations seem to affect a later step in ejection of λ DNA (101). One class of these mutations is called *pel* (penetration of λ) which allow normal adsorption of phage λ, but only 2 to 10% of the infected cells yield the normal burst progeny phage or form lysogens (156). Phages 434 and 82, which use different outer membrane receptors than phage λ, are also affected by *pel* mutations, indicating that the Pel^- phenotype is not simply due to inactivation of the LamB outer membrane receptor. However, certain other phages (phi 80, T4, P1, and M13) are not affected by *pel* mutations, suggesting that the Pel^- phenotype is somewhat phage specific. In electron micrographs, most λ phage adsorbed to the surface of *pel* $^-$ mutants appear to retain DNA in the phage head, suggesting that the Pel^- phenotype causes a defect in ejection from the phage head. Furthermore, when ^{32}P-labeled λ phage are used to infect a *pel*$^-$ P1 lysogen, the λ DNA is not degraded by the P1 restriction system, indicating that it does not enter the cytoplasm (156).

The *pel*$^-$ mutations map in the *manXYZ operon*, which encodes proteins II-P of the mannose phosphotransferase system (42, 45, 127). The *pel* mutations are alleles of *manY* (5) which encodes the mannose permease. Thus, the mannose permease may be a host factor used by phage λ to translocate its DNA from the outer membrane or periplasmic space into the cytoplasm of the host.

Mutant λ phage were isolated which could infect *pel*$^-$ mutants (157). These mutants fall into two classes, λ *hp* and λ *hp* (Ts). λ *hp* (Ts) mutants are temperature sensitive for infection at 42℃. All of the *hp* (Ts) alleles map in the λ *V* gene (157). The *V* gene encodes the major tail protein (gpV), forming a 135 nm long and 9 nm wide tail tube of 32 hexameric disks with a central hollow core 3 nm in diameter. About 200 copies of gpV are present per phage particle (84). In contrast to the *hp* (Ts) alleles, all of the *hp* alleles map into the λ *H* gene (157). The *H* gene encodes a 90 kDa protein, which is cleaved into a 78 kDa mature form (called H*), which is added to the tail after the gpV tail tube has been polymerized (83, 84). About 6 to 12 copies of the H* protein are present in the phage tail, probably located inside the tail tube. Katsura (83) showed that the middle part of gpH is important in DNA ejection and the stability of the tail tip. It remains to be determined how exactly the structure of gpH is related to its function in DNA ejection and especially how the middle part of gpH, which is not considered to be near to either the head or the tail tip, can influence DNA ejection (83). It has been suggested that cleavage of gpH into H* protein yields a high energy form of the protein that could drive the conformational change in the tail during DNA ejection. The H* proteins seems to be ejected with λ DNA (141, 145). Thus, it has been proposed that H* may function as a “pilot protein”, which mediates transport of the DNA across the cytoplasmic membrane, but this has not been directly

demonstrated (84).

The *Shigella sonnei* LamB receptor, but not the *E. coli* LamB receptor, can trigger spontaneous ejection of DNA from λ *in vitro* (146). *E. coli* can be infected by λ whether the bacteria bear the *Shigella sonnei* LamB receptor or the *E. coli* receptor (142). Therefore, a receptor able to trigger spontaneous ejection of DNA *in vitro* is not required for ejection of DNA in bacteria, so some other triggering mechanism must operate *in vivo*. This mechanism is likely to involve the *pel* gene (146).

Wild-type λ infects *E. coli pel*$^-$ strains inefficiently (156). Roa and Scandella (142) showed that the *Shigella sonnei* LamB does not suppress the Pel$^-$ phenotype, a result that was confirmed by Roessner and Ihler (146). Although the phage bind tightly to *pel*$^-$ *E. coli* K12, DNA ejection is blocked (156). Roessner and Ihler (146) showed by electron microscopy that in *pel*$^-$ strains carrying the *Shigella sonnei* LamB protein, infection is also blocked prior to DNA ejection. This result demonstrated that the ability of the *Shigella sonnei* allele to trigger spontaneous ejection is overridden by some other component of the bacterium.

Involvement of the λ tail proteins gpH and gpV in DNA ejection is also likely, since certain mutations in these genes permit infection of *lamB*$^+$ *pel*$^-$ strains (157). Effective DNA ejection by phage λ *in vivo* is dependent on the LamB protein, the Pel protein and the phage proteins gpJ, gpH, and gpV (146).

Xu and Feiss (187) isolated mutations in a specific base pair (λ *cosN-IT*) of the *cosN* site used as a packaging signal by the λ terminase. Although the λ *cosN-IT* phage only have a mild defect in DNA packaging, the mutations reduced infectivity of the phage to 25% of wild-type λ. Electron microscopic observation of *E. coli* infected

with λ *cosN-IT* phage revealed that most of the phage particles retained full phage heads after adsorption, indicating that the phenotype resulted from a failure to eject DNA from the phage. Revertants of *cosN-IT* mutants were either true revertants that restored the original GC base pair, or added another GC base pair next to the mutated site. The interpretation of these results is that the *cosN-IT* mutation does not substantially interfere with positioning of the genome during phage particle assembly and DNA packaging, but rather prevents the proper interaction with a protein in the tail tube which is intimately involved in DNA ejection (187). Xu and Feiss (187) speculate that the GC base pair at the negative one position of *cosN* interacts with the H* protein. Since the λ *hp* mutations map within the *H* gene (157), the H* protein may itself interact with ManY. Thus, the signals for ejection and for DNA transport into the cytoplasm may be directly linked (101).

Edgar *et al*. (41) monitored the infection of *E. coli* cells by light microscopy using phages tagged with quantum dots. Phages were chemically decorated with biotin using a crosslinker that forms a stable amide bond with primary amines. The biotinylated phage was then conjugated to streptavidin-coated quantum dots that are ~10-20 nm in size, smaller than tailed phage particles such as λ. Edgar *et al*. (41) found that at low MOI, most of the infecting phages were found attached to the bacterial poles. This was true for a number of temperate and virulent phages of *E. coli* that use widely different receptors and for phages infecting *Yersinia pseudotuberculosis* and *Vibrio cholerae*. The infecting phages co-localized with the polar protein marker IcsA-GFP. ManY, the *E. coli* protein required for λ ejection, was found to localize to the bacterial poles as well. Furthermore, labeling of λ DNA during infection revealed that it is ejected and replicated at the polar region of infection.

Edgar *et al*. (41) proposed a model for λ infection in which the LamB-bound λ phage migrates along the cell surface until it reaches one of the poles. At the poles, it encounters a “slot” or interacts with additional proteins, which stops its movement. The DNA is ejected at the poles, with the help of ManY, where phage replication occurs (41).

III. Translocation of Phage T7 DNA across the membranes of *Escherichia coli*

Phage T7. The icosahedral head of phage T7 has a diameter of 60 to 61 nm with an outer shell that is composed of two forms of the gene 10 protein (26-28, 39, 148, 168). Inserted at one vertex is the head-tail connector, composed of 12 gp8 molecules (21, 24, 92, 182). A channel, which is closed in mature virions, runs through the center of the connector (36). Inside the head, and attached to the head-tail connector in the coaxial orientation, is a 26 nm X 21 nm cylindrical structure that is usually referred to as the internal core (161, 162). The core has recently been shown to exhibit 8-fold symmetry (25), consists of stacked rings and contains three distinct proteins, the products of genes 14, 15, and 16. The 40-Kb genome is spooled around the internal core in six coaxial shells (23). Unlike most other tailed phages, the T7 tail is not assembled as a separate structure but forms directly on the DNA-filled head (112, 143, 161, 170). The stubby tail is 23 nm long, tapering from a diameter of 21 nm at the connector to 9 nm at its distal end, and is known to consist of two major proteins gp11 and gp12 (170). Attached near the head proximal end of the tail are six symmetrically positioned tail fibers. Each fiber is composed of a trimer of gp17 that forms a kinked structure (80, 81, 167).

Penetration of the outer membrane. Phage T7 initiates an infection of *Escherichia coli* by the interaction of its tail fibers with the lipopolysaccharide (LPS) on the cell surface. Interaction of all six tail fibers with the LPS would orient the phage tail perpendicular to the cell surface, conferring efficiency to subsequent stages of infection. Adsorption of T7 virions requires, in addition to a tail fiber-LPS complex, a productive interaction of at least one of the tail proteins gp11 or gp12 with a cell surface component (86). The stubby T7 tail is too short to span the *E. coli* cell envelope and a channel needs to be made to allow the phage genome to travel from the virion into the cytoplasm. It was proposed that virion proteins are ejected into the cell, functionally endowing T7 with an extensible tail. The major functions of the ejected proteins are to form a channel across the cell envelope, including localized degradation of the cell wall (117, 119), and to translocate the leading end of the phage genome by what appears to be an enzyme-catalyzed reaction (51, 53, 87, 169).

A remarkable series of conformational changes must occur in these ejected proteins at the initiation of infection. The ten copies of gp14, four copies of gp16, and eight copies of gp15 that comprise the internal core structure inside the mature capsid must disaggregate and unfold almost completely in order to pass through the channel (86). The channel presumably formed by the internal core proteins has an average diameter of ~3.7 nm but with a constriction down to ~2.2 nm. The proteins ejected from the T7 capsid must presumably pass through this constricted channel as they travel into the cell (86).

Uptake of DNA into the host cytoplasm. Entry of the leading 850 bp end of the 40 Kb T7 genome occurs by a transcription-independent mechanism (51, 53).

Three promoters for *E. coli* RNA polymerase, A1, A2, and A3, lie at positions 498, 626, and 750 bp from the leading end of the T7 genome (39); one or more of these promoters allows *E. coli* RNA polymerase to internalize about 7 Kb of DNA as the early region of the genome is transcribed. Several T7 promoters and gene 1, coding for T7 RNA polymerase, lie within this 7 Kb and, during a normal infection, T7 RNA polymerase is thought to internalize the remaining 32 Kb of T7 DNA as it transcribes the late region of the genome.

Translocation of phage T7 DNA from the capsid into the cell has been assayed by measuring the time after infection that each GATC site on the phage genome is methylated by cells containing high levels of DNA adenine methylase. Methylation at GATC site on T7 DNA renders both the infecting genome and any newly synthesized molecules sensitive to the restriction enzyme *Dpn*I (51). Knowing the positions of GATC sites on the T7 genome and the time after infection that they become methylated allows an estimate of the rate of genome entry. In a normal infection at 30^{o}C, translocation of the T7 genome into the cell takes between 9 and 12 min. In contrast, translocation of the entire phage λ genome or of a T7 genome ejected from a λ capsid can be detected within the first minute of infection (51). The slow T7 genome entry is part of the mechanism by which T7 avoids restriction by type I restriction enzymes. By requiring transcription by *E. coli* RNA polymerase to internalize the early region of the phage genome, expression of the antirestriction activity of gene 0.3 is ensured (51, 54, 118). By the time sufficient T7 RNA polymerase has accumulated to complete genome transport into the cell, gp0.3 has inactivated the restriction enzyme and restriction sites on DNA that has yet to enter the cell are thus protected from cleavage.

One internal core protein, gp16, has been shown to limit the initial amount of DNA transferred through the channel to ~850 bp. Virions containing one of several mutant gp16 allow the whole genome to enter the cell in the absence of transcription (53, 169). The gp16 mutations all lie within a 380 bp region of the 4 Kb gene 16 and have been shown to be both necessary and sufficient to allow efficient transcription-independent entry of the complete T7 genome. The mutations are thought to inactivate a domain of the protein that clamps on the entering genome, limiting transcription-independent translocation to 850 bp. The mechanism of DNA translocation used by these mutant virions to internalize a complete genome is therefore likely to be the same as that used by the wild-type particle to internalize the initial 850 bp. Using one such mutant virion the whole genome has been shown to enter the cell at a constant rate whose value is temperature-dependent and can be fitted to Arrhenius kinetics. These properties are not those of a process controlled by physical forces; rather, they are properties expected for an enzyme-catalyzed reaction. Kemp *et al*. (87) suggest that one or more proteins of the phage particles form a molecular motor that translocates DNA across the cell envelope. Kemp *et al.* (87) also shows that the proton motive force is necessary for this mode of DNA translocation, because collapsing the proton motive force while the T7 genome is entering the cell abruptly halts further DNA transfer.

IV. Uptake of P22 DNA into the cytoplasm of *Salmonella* Typhimurium

Phage P22. Phage P22 is a temperate, icosahedral, “lambdoid” phage used for generalized transduction of *Salmonella enterica* sv. Typhimurium. It can package up to 43.5 Kb of linear double stranded DNA (dsDNA) and it has a short noncontractile tail (2, 74). Unlike λ and T7, P22 undergoes “head-ful DNA packaging”

which would make it unlikely that the uptake of P22 DNA is transcription-dependent. Like T4, P22 genome is circularly permuted and about 1.8 Kb of DNA is repeated at both ends of the DNA molecule (74). P22 also uses the host RNA polymerase for transcription of all its genes.

Reversible adsorption. During P22 infection of its bacterial host, the first event that happens is the recognition of *Salmonella* Typhimurium by P22. This recognition is mediated by the reversible adsorption of the phage to the O-Antigen (O-Ag) on the outer membrane of *Salmonella* Typhimurium (101). The phage tail fibers which are composed of gp9 proteins are specific to the O-Ag of *Salmonella* Typhimurium. The tail fibers also have endorhamnosidase activity (44, 71) that cleaves between the rhamnose and galactose moieties of the O-Ag of the LPS during reversible adsorption which exposes the secondary receptor on the outer membrane of *Salmonella* Typhimurium (44, 71).

Irreversible adsorption. Irreversible adsorption occurs when the phage tail spike which is composed of the gp26 protein binds to the secondary receptor (71). An unknown mechanism triggers the release and ejection of the phage DNA into the periplasmic space (70). The phage-encoded pilot proteins are thought to protect the DNA from degradation by nucleases in the periplasm and these pilot proteins are also suspected of guiding the DNA across the cytoplasmic membrane (12, 70, 179).

Upon entry of the phage DNA into the host cytoplasm, the linear dsDNA circularizes by recombination of its redundant terminal ends. At this point, the prophage either integrates into the bacterial chromosome or produces progeny phage and lyses the bacterial cell. If it proceeds as a lytic phage, it will undergo between five

to ten rounds of theta replication and express phage proteins required for assembly of phage heads and tails. It switches to rolling-circle replication and starts packaging phage DNA. Progeny phage accumulate and enzymes are expressed that lyse the bacterial cell.

Transport of DNA across the cytoplasmic membrane. Although a great deal is known about the life cycle of P22, the mechanism of DNA transport is poorly understood. The main question that this project aims to address is how phage DNA crosses the cytoplasmic membrane. The “hypodermic injection” model that was incorrectly proposed for phages with long tails like T4 (65) also cannot provide a satisfactory explanation on how the DNA of P22 gets into the host cytoplasm because P22 has a short noncontractile tail that cannot reach the cytoplasmic membrane of the host. Gene 26 of P22 that encodes for the tailspike protein is only 21 nm in length (2) while the distance from the outer membrane to the cytoplasmic membrane of the bacterial host is about 33 nm (38). The outer membrane is approximately 14 nm thick (124) and the cytoplasmic membrane is between 5-6 nm thick (62, 108). The tail spike of P22 would have to be at least 52.4 nm in length to be able to inject its DNA directly into the host cytoplasm. The tail spike of P22 is short by roughly 31 nm.

Furthermore, a number of convincing evidence provided by Susskind *et al*. (173) and by Israel (70) indicate that the phage DNA does not get transported directly into the host cytoplasm. The phage DNA is initially ejected from the phage capsid into the periplasmic space and then transported across the cytoplasmic membrane. Elaborate experiments by Susskind, Botstein and Wright showed that although superinfecting phage P22 adsorbed to P22 *sieA*$^{+}$ lysogens, ejection of superinfecting DNA was apparently incomplete, since the DNA did not become available to the host

DNA restriction system (173). Although it was not extensively degraded, much of the SieA-excluded DNA was nicked, indicating that at least some of it was ejected from the phage head.

The gp7, gp16, and gp20 proteins are required for transport of DNA from P22, including both P22 DNA and transducing DNA (101). Null mutations in any of the three genes result in non-infective phage, although the morphology of the phage particles is not affected (17, 131). Israel (70) reported that the defective phage undergo irreversible adsorption and DNA was ejected from some of the phages, but the DNA did not enter the cell (70). Based on this finding, Israel speculated that after ejection into the periplasm, the phage-encoded pilot proteins (gp 7, 16, and 20) may function in its transport across the cytoplasmic membrane. The ability of $gp16^{+}$ phage P22 particles with UV-irradiated DNA to complement $gp16^{-}$ particles *in trans* also led Thomas and Prevelige to propose that gp16 functions as a pilot protein to facilitate transport of the phage DNA by forming a pore in the cytoplasmic membrane (179).

The sequence of gp20 is not homologous with any entries in the database and the hydropathy plot does not bare any information to suggest function (1). The sequence of gp16 shows no evidence for α-helical membrane spanning domains (181), but gp16 might polymerize *in vivo* (179) to form amphipathic α-helices or multimeric β-strand pores like those of the porins (56). The hydropathy plot of gp7 led Conlin *et al*. (29) to propose that gp7 may function as a pilot protein where the basic N-terminus of the protein interacts with DNA and the hydrophobic C-terminus interacts with the cytoplasmic membrane. Alternatively, the mature N-terminus of gp7

may interact with the cytoplasmic membrane and promote insertion of the C-terminus into the membrane (29).

Superinfection exclusion. P22 lysogens express the c2, the SieB and the SieA proteins to prevent superinfection by related phage (101). Like the λ cI protein, the P22 c2 protein also prevents growth of superinfecting P22 and related phages with similar *immC* regions by repressing lytic gene transcription (130). The SieB protein inhibits the lytic development of certain superinfecting phages by aborting macromolecular synthesis; however, P22 expresses the Esc protein which bypasses the effect of the SieB protein (174, 175). The SieA protein excludes superinfecting DNA at an earlier stage and the exclusion is stronger than SieB (40, 173).

The *sieA* gene has been sequenced (66). Although the SieA protein seems to localize at the cytoplasmic membrane, the function of the SieA protein is still not known. The superinfecting DNA in *sieA*$^+$ lysogens remains trapped in the periplasmic space where it is rapidly degraded by endogenous nucleases (173). Based on these experiments, it has been proposed that SieA may inactivate the DNA transport apparatus by interaction with an unknown component at the cytoplasmic membrane (101). The SieA exclusion may be due to inactivation of one of the P22 pilot proteins. Alternatively, superinfection exclusion may inactivate a host component which interacts with one or more of these P22 proteins (101).

Summary. There is a great deal of information about the life cycle of phage P22, but very little about the transport of phage DNA across the host cytoplasmic membrane. The goal of this project is to use P22 and *Salmonella* as the model to

study how phage DNA is transported across the cytoplasmic membrane of the bacterial host.

Chapter 2. Transport of phage P22 DNA across the cytoplasmic membrane

Abstract

Although a great deal is known about the life cycle of bacteriophage P22, the mechanism of phage DNA transport into *Salmonella* is poorly understood. P22 DNA is initially ejected into the periplasmic space and subsequently transported into the host cytoplasm. Three phage-encoded proteins (gp16, gp20, and gp7) are ejected with the DNA. To test the hypothesis that one or more of these proteins mediate transport of the DNA across the cytoplasmic membrane, we purified gp16, gp20, and gp7 and analyzed their ability to associate with membranes and to facilitate DNA uptake into membrane vesicles *in vitro*. Membrane association experiments revealed that gp16 partitioned into the membrane fraction while gp20 and gp7 remained in the soluble fraction. Moreover, addition of gp16, but not gp7 or gp20 to liposomes preloaded with a fluorescent dye promoted release of the dye. Transport of ^{32}P-labeled DNA into liposomes occurred only in the presence of gp16 and an artificially-created membrane potential. Taken together, these results suggest that gp16 mediates the active transport of P22 DNA across the cytoplasmic membrane of *Salmonella*.

Key words: Bacteriophage P22, DNA uptake, *Salmonella*

Introduction

Phage T2 and T4 are commonly depicted in textbook images performing a "hypodermic syringe-like" mechanism to eject its DNA into *E. coli* (59, 65). This led to the idea that phage DNA is directly injected into the host cytoplasm by the contraction of the phage tail and by the pressure built up in the phage head (59). T4 DNA is probably not injected directly into the host cytoplasm since the tail tube cap seems only to reach the outer surface of the cytoplasmic membrane after tail sheath contraction (57, 164). Thus, the pressure that exists inside the phage capsid due to DNA compression (46, 134) is most likely used by phages to eject their DNA into the periplasmic space of the host. Moreover, the osmotic pressure of the bacterial cytoplasm exerts an opposing force preventing complete transfer of phage DNA *in vivo* (59). The fact that phage T7 DNA enters the cell at a constant rate is also incompatible with a simple pressure-driven process (52, 53, 59).

Phage P22 is a temperate, icosahedral, "lambdoid" bacteriophage that is commonly used for generalized transduction in *Salmonella enterica* sv. Typhimurium. Aside from being noncontractile, P22 has a much shorter tail than phage T4 which cannot penetrate both the outer and inner membrane of its host. Therefore, the pressure inside the phage head of P22 can only be used to eject the P22 DNA into the periplasmic space of the host.

The P22 gene 9 protein forms the hexameric tailspike that specifically recognizes the *Salmonella* O-Antigen. After reversible binding to the O-Antigen, the endorhamnosidase activity of the tailspike proteins cleaves the O-antigen subunits of the lipopolysaccharide (LPS) until it recognizes an uncharacterized secondary

receptor on the outer membrane of the host bacterium. Binding to the secondary receptor triggers release of the phage DNA together with the phage-encoded ejection proteins (gp7, gp16, and gp20) into the periplasmic space of the host (70).

The phage-encoded ejection proteins are essential for the viability of the phage. One of the proposed functions of the ejection proteins is to protect the phage DNA from degradation by nucleases in the periplasmic space. In addition to protecting the DNA from degradation, the phage-encoded ejection proteins may target the DNA to the cytoplasmic membrane and facilitate transport into the cytoplasm (12, 70, 179).

If the ejection proteins direct the translocation of the phage DNA across the cytoplasmic membrane, at least one of these proteins would be expected to interact with the lipid or a protein in the outer leaflet of the *Salmonella* cytoplasmic membrane. Simultaneously binding the phage DNA and associating with the inner membrane would allow the ejection proteins to target and anchor the phage DNA to the inner membrane of the host, as an initial step in transport of the phage DNA into the cytoplasm.

The hydropathy plot of gp7 led Conlin *et al*. (29) to propose that gp7 may function as an ejection protein where the basic N-terminus of the protein interacts with DNA and the hydrophobic C-terminus interacts with the cytoplasmic membrane. The sequence of gp20 is not homologous with any entries in the database and the hydropathy plot does not reveal insights that suggest function (1). Umlauf and Dreiseikelmann reported an absence of bioinformatic evidence for α-helical membrane spanning domains (181), but gp16 might polymerize *in vivo* (179) to form

amphipathic α-helices or multimeric β-strand pores like those of the porins (56). The purpose of this study was to investigate the role of the ejection proteins in the active transport of phage P22 DNA across the host cytoplasmic membrane of *Salmonella*.

Materials and Method

Plasmids and strains. The pET30 Enterokinase/Ligation-Independent Cloning (Ek/LIC) vector, pCDF Ek/LIC, pET46 Ek/LIC, GST adaptor, NovaBlue cells, and the BL21 *E. coli* expression host cell were purchased from Novagen. The plasmids with the appropriate inserts were maintained in NovaBlue *E. coli* cells {*endA1 hsdR17* (r_{K12}^{-} m_{K12}^{+}) *supE44 thi-1 recA1 gyrA96 relA1 lac* F′[*proA*$^{+}$*B*$^{+}$ *lacI*^{q}*lacZdelM15*::Tn*10*] (Tet^{R})}.

Chemicals. 1,2-dilauryl-*sn*-glycerol-3-phosphatidylcholine was obtained from Calbiochem. 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), Isopropyl-β-D-thiogalactoside (IPTG), lysozyme, sucrose, Triton X-114, chloroform, ether, proteinase K, potassium chloride, and Triton X-100 were purchased from Fisher Scientific (Pittsburgh, PA). Calcein was purchased from Sigma. Rabbit anti-6xHis and mouse anti-GST antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Goat anti-rabbit and goat anti-mouse antibodies were purchased from Jackson ImmunoResearch (West Grove, PA). DNase I was purchased from Fermentas (Glen Burnie, MD). His-bind affinity column purification kit, S*tag agarose, mouse anti-S*tag antibody, protein G/A agarose, enterokinase and benzonase were purchased from Novagen (San Diego, CA). The BCA Protein Assay kit used to determine protein concentration and the GST resin were obtained from Pierce Biotechnology, Inc (Rockford, IL).

Vector Construction. DNA sequences of genes coding for the P22 pilot proteins (gp 7, 16, and 20) were obtained from genbank. The P22 DNA template was extracted from concentrated P22 lysate as previously described (111). Forward and reverse primer sequences used to amplify gene 7 were: forward (5' to 3') GACGACGACAAGATGAAAGGCGGTAAAGGTGGCGCAGATAAAAGC; reverse (5' to 3') GAGGAGAAGCCCGGTTTAAAACAACGAGCCAAGCAGACCAATACC. Forward and reverse primer sequences used to amplify gene 16 were: forward GACGACGACAAGATGAAAGTTACCGCTAATGGCAAGACATTC; reverse GAGGAGAAGCCCGGTCTACTGCCGGGTAGCTTCGTTAGCTAAAAG. Forward and reverse primer sequences used to amplify gene 20 were: forward GACGACGACAAGATGGCTACGTGGCAGCAGGGCATTAATTCAGGT; reverse GAGGAGAAGCCCGGTTTATTCCACCGTGAATTTAATGCCAGATTT.

The amplified gene sequences were cloned into the pET30 Ek/LIC, pCDF Ek/LIC, or pET46 Ek/LIC expression vectors from Novagen. The presence of the appropriate inserts was confirmed by PCR, restriction digests and DNA sequencing. Expression vectors with the correct inserts were electroporated into the expression host cell line BL21 from Novagen, and then plated on LB agar plates with the appropriate selection marker (50 µg/ml Kanamycin for pET30, 100 µg/ml Spectinomycin for pCDF, and 100 µg/ml Ampicillin for pET46).

To study the interaction between gp20 and gp16, the S*tag DNA sequence from the pCDF vector harboring the gp20 insert was removed using *Bsu*36I and *Xho*I.

Protein Expression and Purification. BL21 cells with each plasmid clone were inoculated into 100 ml of LB broth with the appropriate antibiotic concentration

and incubated in a 37°C shaker until mid-exponential growth. IPTG was added to a final concentration of 1 mM to induce protein expression and the cultures reincubated for an additional 3 hr in a 30°C shaker. The cells were then centrifuged at 3842 x g for 30 min at 4°C. The supernatant was removed and the pellet resuspended in 4 ml of lysis buffer (50 mM NaH_2PO_4, pH 8, 300 mM NaCl, 10 mM Imidazole, 10 mM CHAPS). Lysozyme was added to 0.5 mg/ml and 100 units of benzonase was also added to reduce viscosity of the lysate. The lysate was incubated in ice for 30 min with occasional swirling and then subjected 3 times to sonication using the Fisher Scientific ultrasonic dismembrator Model 100. The sonicated lysate was centrifuged at 11000 x g for 30 min at 4°C. The supernatant was added to a column containing 200 µl bed volume of nickel-charged His-bind resin (Novagen) and samples eluted at a flowrate of ca. 1 ml/min. The resin was washed three times with 1X binding buffer (50 mM NaH_2PO_4, pH 8, 300 mM NaCl, 20 mM Imidazole, 10 mM CHAPS) and washed twice with 1X wash buffer (50 mM NaH_2PO_4, pH 8, 300 mM NaCl, 60 mM Imidazole, 10 mM CHAPS) before the 6xHis-tagged ejection protein was eluted three times with 200 µl each of 1X elution buffer (50 mM NaH_2PO_4, pH 8, 300 mM NaCl, 1 M Imidazole, 10 mM CHAPS). The 1 M imidazole was removed from the eluted ejection protein by using a centricon filter with a molecular weight cut off of 5 kDa and by washing the ejection protein with 20 mM Tris buffer, pH 8, 100 mM NaCl. The purified proteins were treated with enterokinase according to manufacturer's instructions to remove the N-terminal 6xHis tag prior to using the purified proteins in the DNA binding, membrane disruption, and DNA transport assays. Protein concentration was determined using the BCA Protein Assay kit from Pierce. Alternatively, GST-tagged proteins were purified using an affinity column packed with

the GST resin. 1X phosphate buffered saline (PBS) was used as the wash buffer for the GST column and 10 mM reduced Glutathione was used to elute the GST-tagged proteins.

Co-elution and co-immunoprecipitation. 6xHis-tagged and GST-tagged proteins were co-expressed in the same BL21 expression host cell and either the 6xHis-tagged protein or the GST-tagged protein was eluted using a nickel-charged His-bind resin (200 µl bed volume) or a GST resin (200 µl bed volume), respectively. The co-eluted protein was detected using the rabbit anti-6xHis or the mouse anti-GST. To immunoprecipitate the tagged proteins, 25 µg of the primary antibody was added to the cleared lysate and incubated for 2 hr with gentle rocking at 4^{o}C. This was followed by the addition of 40 µg of protein G/A agarose and rocking was continued for another hour at 4^{o}C before the samples were centrifuged for 5 min in a clinical centrifuge to pellet the protein G/A agarose. After extensive washing with a buffer consisting of 20 mM Tris, 100 mM NaCl, 0.1% Triton X-100, the protein G/A agarose was boiled in 6x Laemmli dye for 5 min at 99^{o}C and the samples were separated on a 12% SDS polyacrylamide gel. The proteins were transferred on a polyvinylidene fluoride (PVDF) membrane and the co-immunoprecipitated protein was detected using the rabbit anti-6xHis or the mouse anti-GST antibody.

Western Blot. Primary antibodies were used at 1:1500 dilution. The goat anti-rabbit and goat anti-mouse secondary antibodies conjugated with horseradish peroxidase were used at 1:10,000 and 1:5,000 dilution, respectively.

Labeling of DNA with ^{32}P. The *bla* DNA was PCR-amplified from the pUC19 vector by using the following forward and reverse primers: 5'-GACGACGACAAGATGAGTATTCAACATTTCCGTGTCGCCCTTATTCCC-3'

5'-GAGGAGAAGCCCGGTTTACCAATGCTTAATCAGTGAGGCACCTATCTC-3'. The PCR product was 5' end labeled using T4 polynucleotide kinase (New England Biolabs) and 20 µCi [γ-^{32}P]ATP (154).

Gel Retardation Assays. Varying concentrations of the purified pilot proteins were incubated with a final concentration of 1 nM ^{32}P-*bla* DNA for 1 hr at 4^{o}C. All reaction mixtures were in 20 mM Tris, 100 mM NaCl, pH 8 in a total volume of 36 µl. Reactions were mixed with a sucrose dye solution (153) and separated on a 5% native polyacrylamide gel. Vacuum-dried gels were exposed to a phosphorimager screen and analyzed using Molecular Dynamics ImageQuant v. 5.2 software. A final concentration of 12 nM of salmon sperm DNA was added to the preincubated mixture of pilot protein and ^{32}P-*bla* DNA to study the DNA-binding specificity of the pilot proteins. When BSA was used in lieu of any of the pilot proteins, the concentration of BSA was equivalent to the pilot protein it was being compared to.

DNA Protection Assays. The procedure for the DNA protection assay is identical to the gel retardation assay except for the incubation of the DNA alone or the DNA with bound pilot proteins with 0.5 unit of DNase I at 37^{o}C for 20 min prior to loading the samples on a 5% native polyacrylamide gel.

Membrane partitioning of pilot proteins. Preparation of the *Salmonella* membrane extracts and the procedure for the membrane partitioning assay was adapted from Muro-Pastor *et al*. (121). Briefly, cells were grown to log phase in 500

ml of LB broth and then centrifuged for 30 min in 4°C at 3842 x g using a Sorvall GSA rotor. The pellet was resuspended in 20 ml of 0.1 M cacodylic buffer, pH 6.8 and then lysed twice using a French pressure cell at 12000 psi. The lysate was centrifuged for 10 min in 4°C at 7818 x g using a Sorvall SS-34 rotor to pellet the cell debris. The supernatant was centrifuged at 227640 x g for 3 hr using a Beckman Vti 65.2 Class H rotor to isolate the membrane fraction. The pellet containing the membrane vesicles was resuspended in 3 ml of 0.1 M cacodylic buffer with 5% glycerol. The membrane extract was passed through a gauge 23 syringe needle at least 5 times prior to performing the assay. A final concentration of 4 µM of the nickel-purified ejection proteins were mixed with 125 µl of the membrane extract in a total volume of 500 µl. $MgCl_2$ was added to a final concentration of 10 mM. The mixture was separated by sucrose gradient ultracentrifugation on a 25% and 65% sucrose layers at 95834 x g for 3 hr using a Ti44 rotor. After centrifugation, the soluble fraction found on top of the 25% layer and the membrane fraction found between the two sucrose layers were loaded on a 10.5% SDS polyacrylamide gel. The proteins in the gel were transferred to a PVDF membrane and probed with the rabbit anti-6xHis antibody.

Triton X-114 Phase separation of the pilot proteins. The Triton X-114 phase separation of the ejection proteins was performed as described by Muro-Pastor *et al*. (121).

Liposome reconstitution. Liposomes with large internal aqueous space or liposomes with encapsulated calcein fluorescent dye were created by reverse-phase evaporation using the procedure described by Szoka and Papahadjopoulos (176). All preparations contained 66 µmol of total lipid per ml of aqueous phase. A thin film of phospholipid was formed on the wall of a round-bottom flask by rotary evaporation of

the chloroform at 42°C. The thin film of phospholipid was resuspended in 3 ml of diethyl ether then 1 ml of 20 mM Tris, pH 7.4, 100 mM NaCl was added to the phospholipid suspension to serve as the aqueous phase encapsulated within the liposomes. When encapsulation of fluorescent dye was desired, 1 ml of 40 mM calcein was added to the aqueous phase. Rotary evaporation of the diethyl ether allowed the thin layer of phospholipid to gradually form liposomes around the aqueous layer. Rotary evaporation was performed for approximately 2 hr at room temperature until all traces of ether had been removed from the sample. The liposomes were then passed through a Sepharose 4B column to remove nonencapsulated material and residual organic solvent. To confirm that the liposomes were intact, an aliquot of the reconstituted liposome fraction was negatively stained and examined under the transmission electron microscope.

Liposomal partitioning assay. A final concentration of 0.15 µM of the nickel-purified ejection proteins were mixed with 66 mM liposomes in a total volume of 150 µl. The mixture of liposomes and ejection proteins were rocked at room temperature for 30 min prior to ultracentrifugation at 227640 x g for 40 min in a TLA-100.1 rotor. The supernatant was transferred to a microcentrifuge tube and the pelleted liposomes were resuspended in 150 µl of 20 mM Tris, pH 7.4, 100 mM NaCl. The soluble and liposomal fractions were boiled in 6x SDS gel sample buffer and loaded on a 12% SDS polyacrylamide gel (153). The proteins were transferred to a PVDF membrane and the membrane was probed with rabbit anti-6xHis antibody to determine where the proteins partitioned.

Membrane-leakage assay. The ability of the pilot proteins to form membrane channels was assayed as described by Zhu *et al*. (190) and Galloux *et al*. (50).

Briefly, calcein was encapsulated inside liposomes. Molecular crowding inside liposomes inhibits calcein fluorescence, but fluorescence is enhanced upon release from the liposomes. A final concentration of 100 nM of the pilot proteins were added to 0.66 mM liposome in a total volume of 100 µl. The samples were excited at 490 nm and the relative fluorescence of each sample was followed for 3 min at an emission wavelength of 510 nm. Total dye release was completed by the addition of 0.2 vol % Triton X-100. Background fluorescence from the liposomes alone was subtracted from the fluorescence reading of each sample. BSA was used as the negative control and ethanol was used as the positive control resulting in complete disruption of the liposomes. The percent relative fluorescence (% RF) for each sample was calculated as follows,

$$\% \text{ RF} = (F_P - F_B) \times 100/(F_T - F_B)$$

where F_P is the fluorescence intensity of the dye released by the protein, F_T is the fluorescence intensity of the total dye released, and F_B is the background fluorescence intensity.

DNA transport assay. The transport of DNA across membranes was studied *in vitro* by following the uptake of ^{32}P-labeled *bla* DNA into liposomes. A final concentration of 100 nM of pilot proteins were added to 1 nM of the radiolabeled DNA. Controls lacking pilot proteins were performed concurrently. The samples were incubated at 4^{o}C for 1 hr to allow the pilot proteins to bind the DNA before adding 20 µl of 66 mM liposome to each sample. The samples were incubated in room temperature for at least 1 hr, then 1 unit of DNase I was added to each sample. The samples were incubated at 30^{o}C for 1 hr to degrade any DNA molecules that were

not transported inside the liposomes then 2 µl of 25 mM EDTA was added to stop the DNase I reaction. The liposomes were centrifuged at 8000 x g for 2 min, and then washed with 20 mM Tris, pH 7.4. The pelleted liposomes were resuspend in 20 mM Tris buffer containing 60 µg of proteinase K and 2% Triton X-100. Samples were separated on a 5% native polyacrylamide gel and the gel was vacuum-dried for phosphorimaging.

Artificial membrane potential across liposomal membrane. Liposomes (66 µmol of total lipid) were reconstituted in 1 ml of buffer solution containing 100 mM KCl, 20 mM Tris, pH 7. The buffer outside the liposomes was changed to 20 mM KCl, 20 mM Tris, pH 7 by carefully pouring the liposomes in a Bio-Rad Poly-Prep chromatography column (Hercules, CA) and equilibrating the column with a buffer containing 20 mM KCl. The column was capped and the liposomes were resuspended in 1 ml of 20 mM KCl, 20 mM Tris, pH 7. Prior to performing the DNA transport assay, 1 µl of 100 mM valinomycin was added to the liposomes to equilibrate the potassium ion concentration inside and outside the liposomes. This creates a net negative charge inside the liposomes with approximately 100 mM Cl^- in the interior and 20 mM Cl^- in the exterior of the liposomes.

Results

Do the pilot proteins interact to form a complex? To assay for protein-protein interactions between the pilot proteins, the 6xHis-tagged gp16, 6xHis-tagged gp20 and the GST-tagged gp7 pilot proteins were expressed in the same *E.coli* expression host by IPTG induction of the expression vectors. The 6xHis-tagged proteins were purified using either a nickel-charged resin in a tube or a nickel-

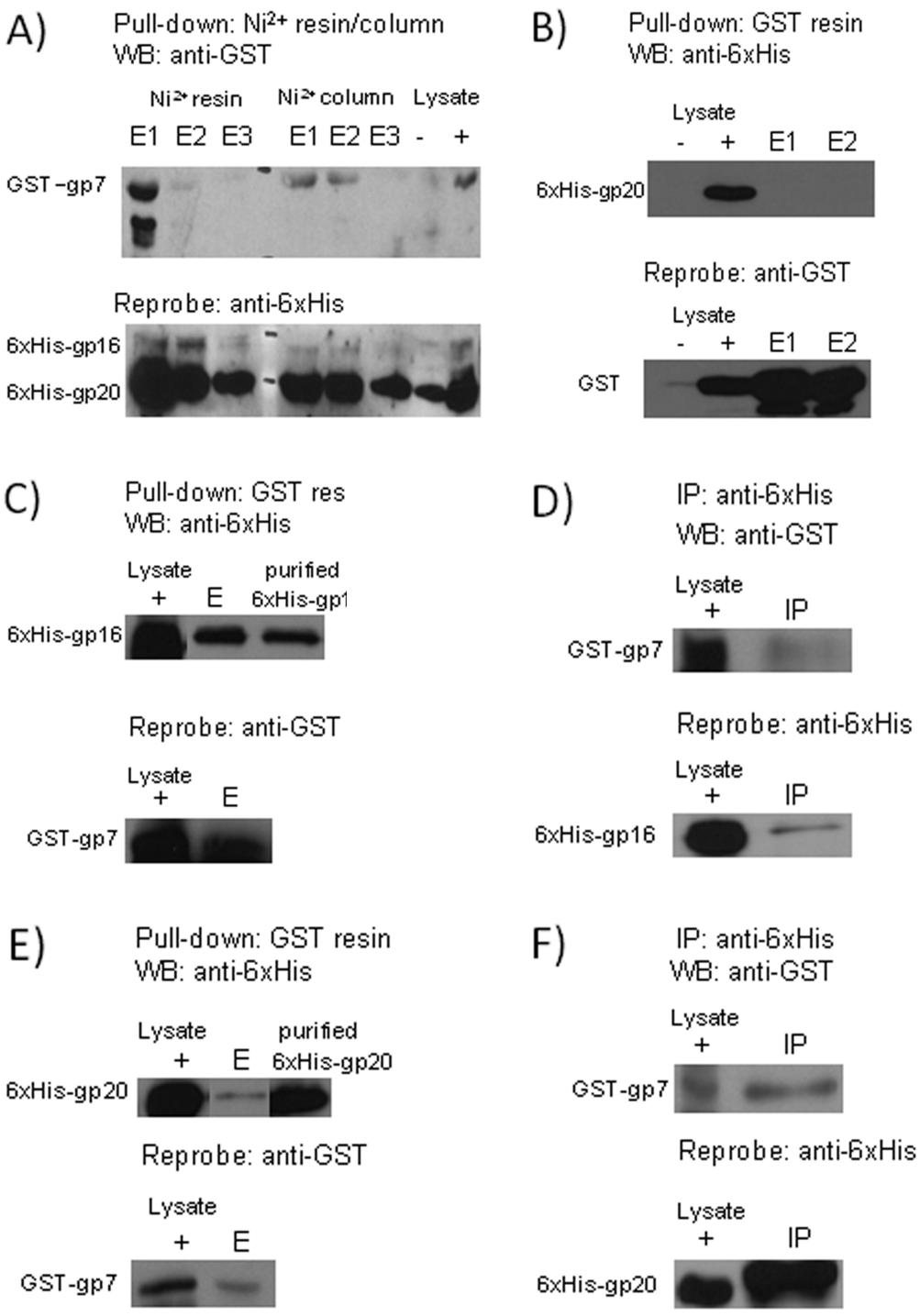
charged resin in a column and the co-eluted GST-gp7 protein was detected using an antibody against the GST tag. The PVDF membrane was reprobed with an antibody against the hexahistidyl tag to confirm the presence of the 6xHis-tagged proteins that co-eluted GST-gp7.

GST-gp7 co-eluted with 6xHis-gp16 and 6xHis-gp20 when both 6xHis-tagged proteins were purified by the nickel resin in a tube or nickel resin in a column (Figure 2.1A). This suggests that 6xHis-gp16, 6xHis-gp20, or both 6xHis-tagged proteins associated with GST-gp7. Reprobing with rabbit anti-6xHis antibody confirmed the presence of the 6xHis-tagged proteins that co-eluted GST-gp7. The expression of 6xHis-gp20 was more robust than 6xHis-gp16 because 6xHis-gp20 was expressed from a higher copy number plasmid than the 6xHis-gp16 expression vector, an experimental condition that mimics the *in vivo* ratio of these pilot proteins in the P22 capsid (100).

As a control, the 6xHis-gp20 and the GST tag were co-expressed in the same host and the GST tag was purified using the GST resin. The presence or absence of 6xHis-gp20 was assayed using the antibody against the hexahistidyl tag. The presence of GST was confirmed by using the antibody against GST. Although 6xHis-gp20 was robustly expressed, 6xHis-gp20 did not co-elute with just the GST tag (Figure 2.1B) indicating that the GST tag was not involved in the association between gp16 and gp7 or gp20 and gp7.

To identify which pilot protein directly binds other pilot proteins, a series of pull down assays was performed in which only two of the pilot proteins were co-expressed

Figure 2.1: *The pilot proteins form a complex*. (A) 6xHis-gp16, 6xHis-gp20 and GST-gp7 were all expressed in the same DE3 *E. coli* cell and the 6xHis-tagged proteins were purified using either a nickel-charged resin in a tube or a nickel-charged resin in a column; the PVDF membrane was probed with mouse anti-GST and then reprobed with rabbit anti-6xHis antibody. (B) 6xHis-gp20 and the GST tag were co-expressed in the same cell and the GST tag was purified using the GST resin. The PVDF membrane was probed with rabbit anti-6xHis antibody and then reprobed with mouse anti-GST antibody. (C) 6xHis-gp16 and GST-gp7 were co-expressed in the same cell and GST-gp7 was purified using the GST resin. The PVDF membrane was probed with rabbit anti-6xHis antibody and then reprobed with mouse anti-GST antibody. (D) 6xHis-gp16 and GST-gp7 were co-expressed in the same cell and 6xHis-gp16 was immunoprecipitated using the rabbit anti-6xHis antibody. Co-immunoprecipitation of GST-gp7 was detected by probing with mouse anti-GST antibody. (E) 6xHis-gp20 and GST-gp7 were co-expressed in the same cell and GST-gp7 was purified using the GST resin. The PVDF membrane was probed with rabbit anti-6xHis antibody and then reprobed with mouse anti-GST antibody. (F) 6xHis-gp20 and GST-gp7 were co-expressed in the same cell and 6xHis-gp20 was immunoprecipitated using the rabbit anti-6xHis antibody. Co-immunoprecipitation of GST-gp7 was detected by probing with mouse anti-GST antibody. (G) 6xHis-gp20 and 6xHis-S*tag-gp16 were co-expressed in the same cell and 6xHis-S*tag-gp16 was purified using the S*tag resin. The PVDF membrane was probed with rabbit anti-6xHis antibody. (H) 6xHis-gp20 and S*tag were co-expressed in the same cell and the S*tag was purified using the S*tag agarose. The PVDF membrane was probed with rabbit anti-6xHis antibody and then reprobed with mouse anti-S*tag antibody. WB = Western blot, IP = immunoprecipitated or pulled down, (+) lysate = IPTG-induced lysate, (-) lysate = uninduced lysate, E1, E2, E3 = first, second, and third elutions.


A)
Pull-down: Ni2+ resin/column
WB: anti-GST
Ni2+ resin
Ni2+ column
Lysate
E1 E2 E3 E1 E2 E3 - +
GST-gp7
Reprobe: anti-6xHis
6xHis-gp16
6xHis-gp20
B)
Pull-down: GST resin
WB: anti-6xHis
Lysate
- + E1 E2
6xHis-gp20
Reprobe: anti-GST
Lysate
- + E1 E2
GST
C)
Pull-down: GST res
WB: anti-6xHis
Lysate
+ E
purified
6xHis-gp1
6xHis-gp16
Reprobe: anti-GST
Lysate
+ E
GST-gp7
D)
IP: anti-6xHis
WB: anti-GST
Lysate
+ IP
GST-gp7
Reprobe: anti-6xHis
Lysate
+ IP
6xHis-gp16
E)
Pull-down: GST resin
WB: anti-6xHis
Lysate
+ E
purified
6xHis-gp20
6xHis-gp20
Reprobe: anti-GST
Lysate
+ E
GST-gp7
F)
IP: anti-6xHis
WB: anti-GST
Lysate
+ IP
GST-gp7
Reprobe: anti-6xHis
Lysate
+ IP
6xHis-gp20

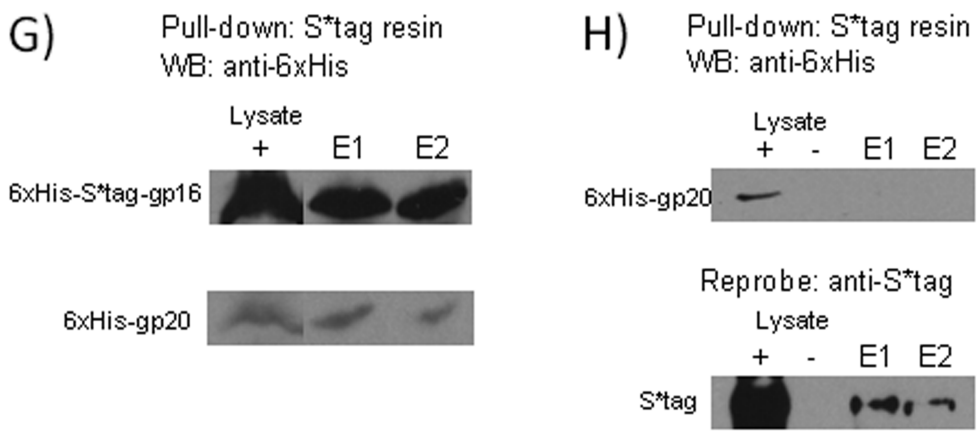


Figure 2.1: *The pilot proteins form a complex*, Continued.

at a time. The specific interaction between gp16 and gp7 was investigated first by purifying GST-gp7 and then detecting the co-elution of 6xHis-gp16 using an antibody against the hexahistidyl tag. 6xHis-gp16 co-eluted with GST-gp7 when the GST resin was used to elute GST-gp7 (Figure 2.1C).

To confirm that gp16 interacts with gp7, the reverse co-immunoprecipitation experiment was performed (Figure 2.1D). This time 6xHis-gp16 was immunoprecipitated using an antibody against the 6xHis tag and the co-immunoprecipitation of GST-gp7 was detected using the mouse anti-GST antibody. The membrane was reprobed with the rabbit anti-6xHis to confirm the presence of 6xHis-gp16. The results shown in Figure 2.1D confirm the interaction between gp16 and gp7.

An analogous pull down assay was done with gp20 and gp7. GST-gp7 was purified using the GST affinity column and the co-elution of 6xHis-gp20 was detected using the anti-6xHis antibody. As with gp16 and gp7, 6xHis-gp20 co-elutes with GST-

gp7 (Figure 2.1E). The reverse co-immunoprecipitation experiment confirms that GST-gp7 interacts with 6xHis-gp20 (Figure 2.1F).

To examine if gp16 can interact with gp20, gp16 was tagged with both the hexahistidyl tag and the S*tag while gp20 was tagged only with the hexahistidyl tag. 6xHis-S*tag-gp16 was purified using the S*tag resin and the co-elution of 6xHis-gp20 was detected using the rabbit anti-6xHis antibody. 6xHis-S*tag-gp16 interacted with 6xHis-gp20 when 6xHis-S*tag-gp16 was purified by the S*tag resin (Figure 2.1G).

To rule out the possibility that 6xHis-gp20 associates with the S*tag and not the gp16 component of the fusion protein, 6xHis-gp20 and the S*tag were co-expressed in the same host and the co-elution of 6xHis-gp20 was tested when the S*tag was purified using the S*tag resin. Purifying the S*tag using the S*tag resin was unsuccessful in co-eluting 6xHis-gp20 (Figure 2.1H). This data shows that there is no interaction between the S*tag and 6xHis-gp20 which suggests that 6xHis-gp20 interacts with the gp16 component of the 6xHis-S*tag-gp16 fusion protein.

Taken together, these results suggest that each of the three pilot proteins interact, possibly forming a Pilot Protein Complex (PPC).

Do the pilot proteins bind and protect the DNA from degradation? The pilot proteins are critical for the survival of the phage DNA while in the periplasmic space. It has been proposed that the pilot proteins bind to the phage DNA to protect it from periplasmic nucleases (12, 172, 179). Because pilot proteins are required for uptake of generalized transducing fragments as well as phage DNA, the DNA binding is expected to be nonspecific.

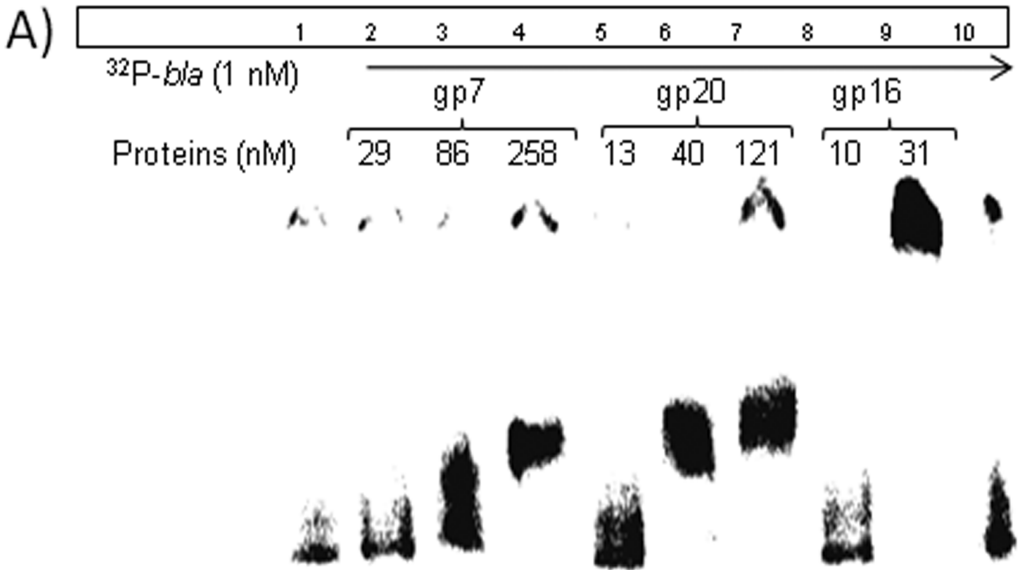
DNA binding of pilot proteins to phage DNA was initially assayed via a gel retardation assay. As a convenient nonspecific DNA fragment, the *bla* gene was PCR-amplified from the pUC19 vector and the PCR product was 5' end labeled with ^{32}P. Increasing amounts of the different pilot proteins were added to the radiolabeled DNA then the samples were separated by PAGE. As shown in Figure 2.2A, all three pilot proteins bound the radiolabeled DNA in a concentration-dependent manner.

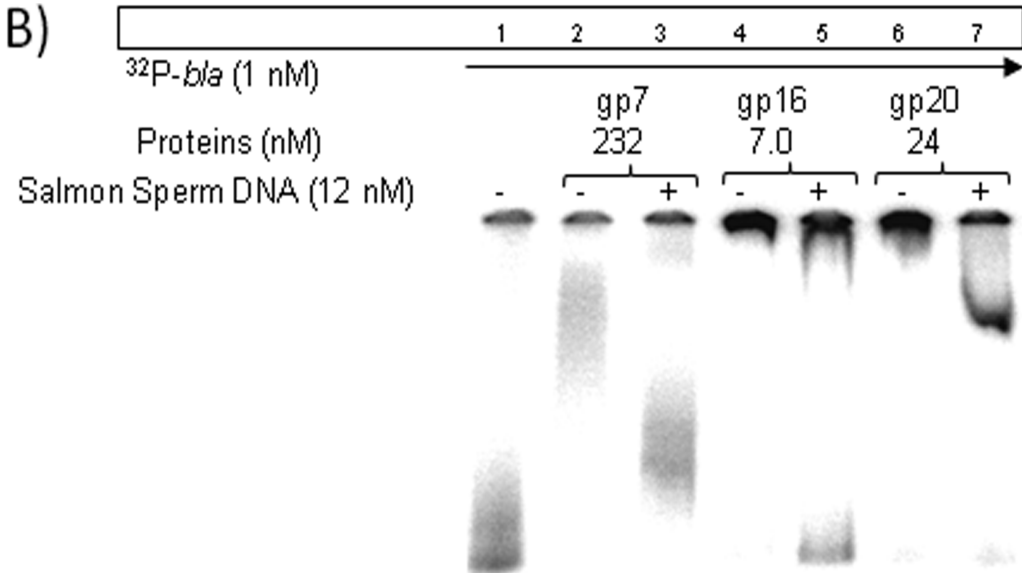
To confirm that the pilot proteins bind DNA non-specifically, a final concentration of 12 nM salmon sperm DNA was added as a competitive non-radiolabeled DNA to prebound pilot protein - ^{32}P-labeled *bla* DNA complex. Figure 2.2B shows that the radiolabeled DNA bound to each of the pilot proteins can be dislodged by nonspecific competition.

To assess whether the pilot proteins can protect DNA from nucleases, the radiolabeled DNA was subjected to DNase I in the presence or absence of the different pilot proteins. Lane 8 in Figure 2.2C shows that 0.5 unit of DNase I was sufficient to completely degrade the radiolabeled DNA in 37^{o}C for 20 min. The presence of any of the pilot proteins protected the radiolabeled DNA from degradation by DNase I as seen in lanes 3, 5 and 7. To demonstrate that protection from DNase I was not simply due to nonspecific protection by the proteins, BSA was tested for its ability to bind and protect DNA from DNase I. Unlike the pilot proteins, BSA was unable to protect the DNA from degradation by DNase I (Figure 2.2D).

Do the pilot proteins associate with membrane? If the pilot proteins facilitate transport of DNA across the cytoplasmic membrane, at least one of the pilot proteins must associate with the *Salmonella* cytoplasmic membrane. To test whether

Figure 2.2: *P22 pilot proteins bind ^{32}P-bla DNA*. (A) 1 nM of ^{32}P-*bla* DNA was added to all reaction tubes with different concentration of pilot proteins. Samples were run on a 5% native polyacrylamide gel and the radiolabeled DNA was detected using a Molecular Probe phosphoimager. Lanes 1 and 10 are unbound radiolabeled DNA. Lanes 2 - 4 are samples of radiolabeled DNA with increasing concentration of the gp7 protein; lanes 5 - 7 are samples of radiolabeled DNA with increasing concentration of the gp20 protein; lanes 8 - 9 are samples of radiolabeled DNA with increasing concentration of the gp16 protein. (B) The pilot proteins bind DNA non-specifically. A final concentration of 12 nM of the competitive non-radiolabeled salmon sperm DNA was added to radiolabeled *bla* DNA pre-incubated with the pilot proteins. Lane 1 shows the mobility of the unbound radiolabeled DNA. (C) The pilot proteins protect the ^{32}P-*bla* DNA from degradation by DNase I. Lanes 1 and 9 show the mobility of the unbound radiolabeled DNA. Lane 8 shows the complete degradation of the radiolabeled DNA after incubation with 0.5 unit of DNase I at 37^{o}C for 20 min. Lanes 2, 4 and 6 are samples containing the radiolabeled DNA with the different pilot proteins. Lanes 3, 5 and 7 are samples containing the radiolabeled DNA with the different pilot proteins incubated with 0.5 unit of DNase I at 37^{o}C for 20 min. No or little degradation of the radiolabeled DNA was seen when the DNA was incubated with any of the pilot proteins. D) BSA does not bind and protect DNA. Lane 4 shows the mobility of the unbound radiolabeled DNA. Lane 3 is the radiolabeled DNA incubated with 0.5 unit of DNase I at 37^{o}C for 20 min. Sample in lane 2 contains the radiolabeled DNA with gp7 incubated with 0.5 unit of DNase I at 37^{o}C for 20 min. Sample in lane 1 contains the radiolabeled DNA with an equivalent concentration of BSA incubated with 0.5 unit of DNase I at 37^{o}C for 20 min.


A)
1 2 3 4 5 6 7 8 9 10
32P-bla (1 nM)
gp7
gp20
gp16
Proteins (nM)
29 86 258 13 40 121 10 31


B)
1 2 3 4 5 6 7
32P-bla (1 nM)
gp7
gp16
gp20
Proteins (nM)
232
7.0
24
Salmon Sperm DNA (12 nM)
- - + - + - +

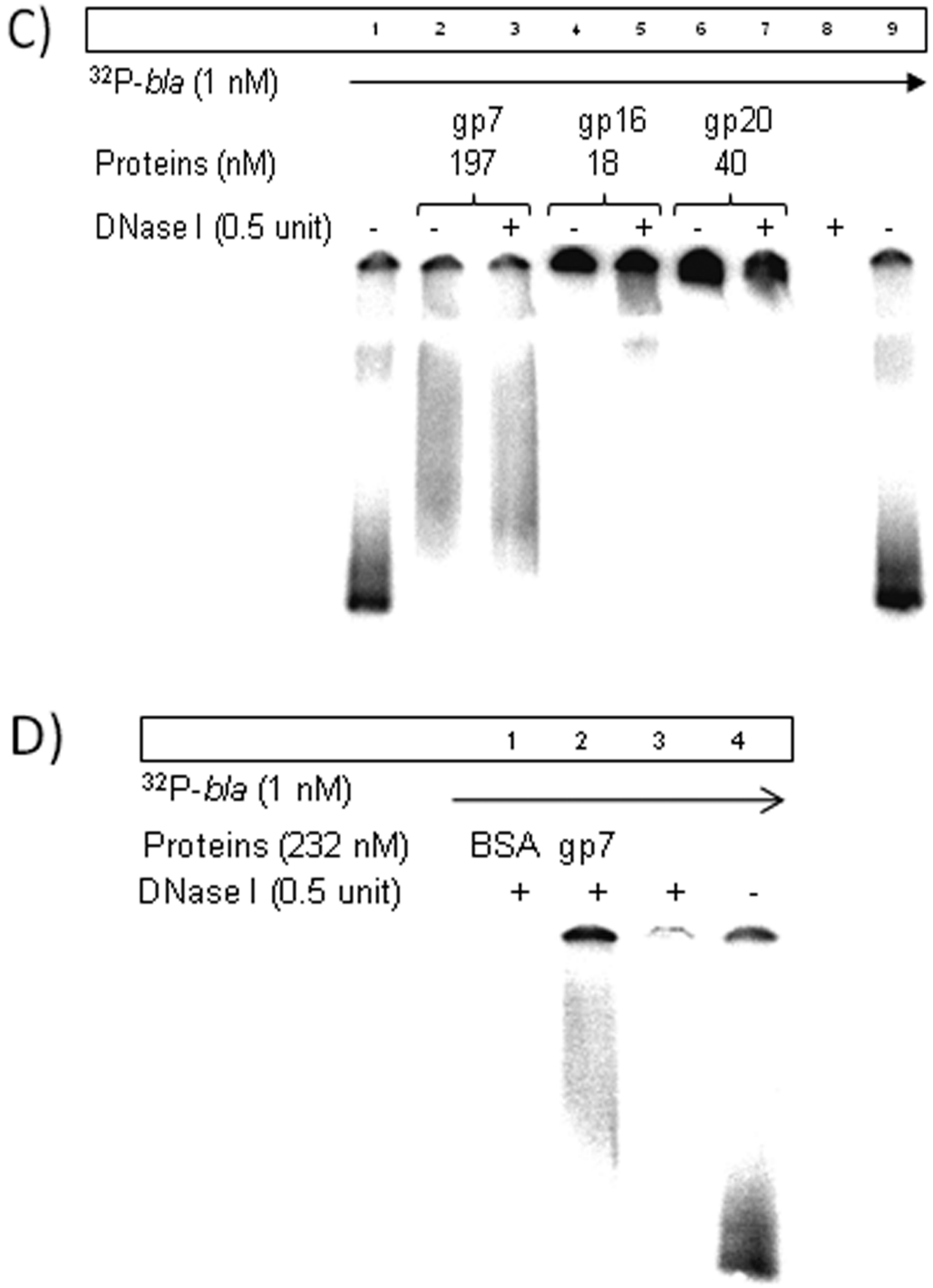
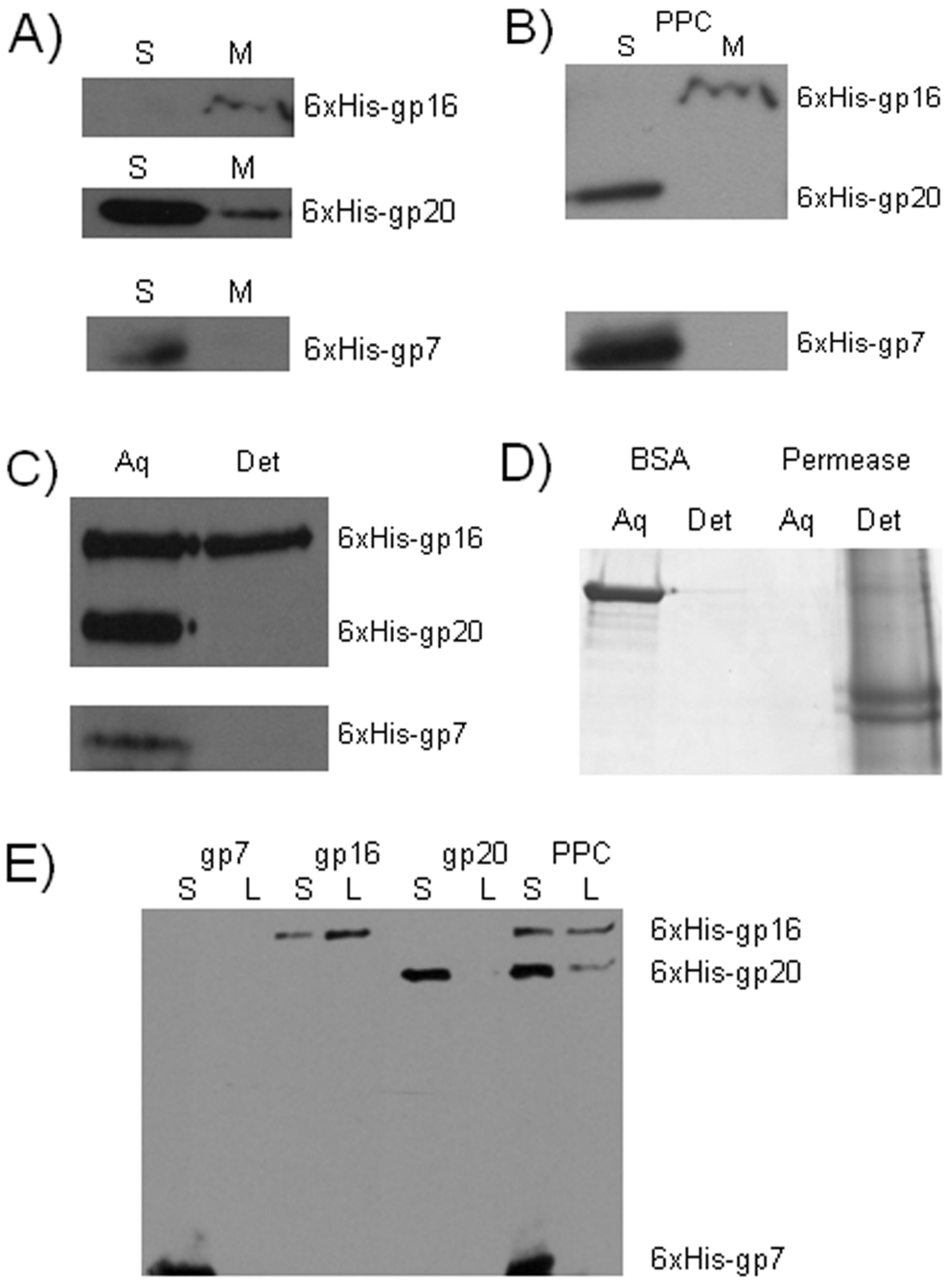


Figure 2.2: *P22 pilot proteins bind ^{32}P-bla DNA*, Continued.

any of the pilot proteins or if the Pilot Protein Complex (PPC) can partition into the cytoplasmic membrane, a membrane partitioning experiment was conducted. Purified pilot proteins or pre-formed Pilot Protein Complex were added to membrane extracts purified from *Salmonella* Typhimurium. The pilot proteins were allowed to equilibrate between the soluble (S) and the membrane (M) fractions at room temperature for 30 min, and then the samples were separated on a sucrose step gradient. The material on top of the 25% sucrose contained the soluble fraction and the material between the two layers contained the membrane fraction (121). The soluble and membrane fractions were separated on a 10.5% SDS polyacrylamide gel and the proteins were transferred onto a PVDF membrane for Western blotting with rabbit anti-6xHis. Both the 6xHis-gp16 sample and the PPC sample showed more 6xHis-gp16 protein in the membrane fraction than in the soluble fraction, indicating that 6xHis-gp16 partitions into the membrane fraction (Figure 2.3A and 2.3B). In contrast, 6xHis-gp20 and 6xHis-gp7 remained primarily in the soluble fraction.

The observed partitioning could be due to interactions of gp16 with integral membrane proteins or hydrophobic interactions with membrane lipids. To distinguish these possibilities, Triton X-114 was used in lieu of the membrane extract from *Salmonella*. Proteins mixed with Triton X-114 at 0^{o}C form a homogeneous solution. However, when this solution is incubated at 30^{o}C, it separates into two phases: an upper aqueous phase and a lower hydrophobic phase (15, 35, 133). Partitioning into the hydrophobic phase is characteristic of proteins that interact with membrane lipids. The purified gp16 partitioned into the hydrophobic Triton X-114 phase while gp20 and gp7 partitioned into the aqueous phase (Figure 2.3C). As controls, BSA and the *E. coli* lactose (LacY) permease were used to demonstrate that soluble proteins partition

Figure 2.3: *Membrane partitioning of the pilot proteins*. Membrane partitioning of individual pilot proteins (A) and the Pilot Protein Complex (B) into crude membrane extract from *Salmonella* Typhimurium. Proteins were detected by Western blotting with the rabbit anti-6xHis antibody after transferring the proteins onto a PVDF membrane. S = soluble fraction, M = membrane fraction, PPC = Pilot Protein Complex. (C) Partitioning of the Pilot Protein Complex in TritonX-114 was detected by Western blotting with the rabbit anti-6xHis antibody after transferring the proteins onto a PVDF membrane. Aq = Aqueous phase, Det = Detergent phase (D) Controls demonstrating partitioning of BSA into the aqueous phase and the partitioning of the *E. coli* lactose permease into the detergent phase. Proteins were detected by staining with Coomassie Brilliant Blue. (E) Liposomal partitioning of the pilot proteins. Proteins were detected by probing the PVDF membrane with the rabbit anti-6xHis antibody. S = soluble fraction, L = liposomal fraction. (F) Domain of gp16 responsible for membrane association. Truncated constructs of gp16 were cloned and expressed in the *E.coli* expression host and purified using the nickel column. Each construct was subjected to the liposomal partitioning assay to determine if the construct would partition to the liposomal fraction or remain in the soluble fraction. The different constructs were detected on the PVDF membrane using the rabbit anti-6xHis antibody.


A)
S
M
6xHis-gp16
S
M
6xHis-gp20
S
M
6xHis-gp7
B)
PPC
S
M
6xHis-gp16
6xHis-gp20
6xHis-gp7
C)
Aq
Det
6xHis-gp16
6xHis-gp20
6xHis-gp7
D)
BSA
Permease
Aq
Det
Aq
Det
E)
gp7
gp16
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6xHis-gp16
6xHis-gp20
6xHis-gp7

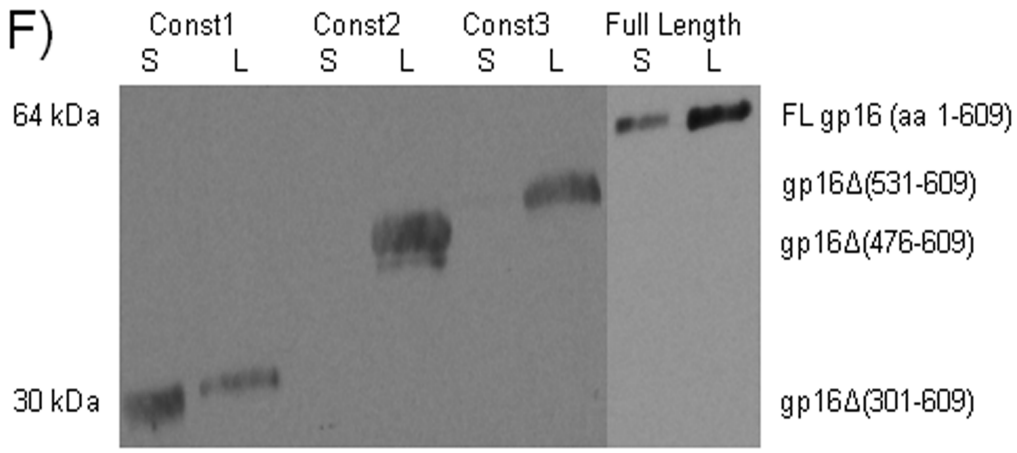


Figure 2.3: *Membrane partitioning of the pilot proteins*, Continued.

in the aqueous phase and that membrane-associated proteins partition in the hydrophobic phase of Triton X-114, respectively (Figure 2.3D).

The membrane-partitioning behavior of the pilot proteins was also confirmed by assaying their association with reconstituted liposomes. The liposomal partitioning assay confirmed the results obtained from the membrane partitioning assay and the partitioning of the pilot proteins in TritonX-114. Gp16 was again found in the liposomal fraction while gp20 and gp7 were primarily in the soluble fraction (Figure 2.3E). The overall behavior of the individual proteins in the liposomal partitioning assay was very similar to their behavior as part of the Pilot Protein Complex except that when the PPC was used, more gp16 protein was found in the soluble fraction and more gp20 was found in the liposomal fraction, possibly due to protein-protein interaction.

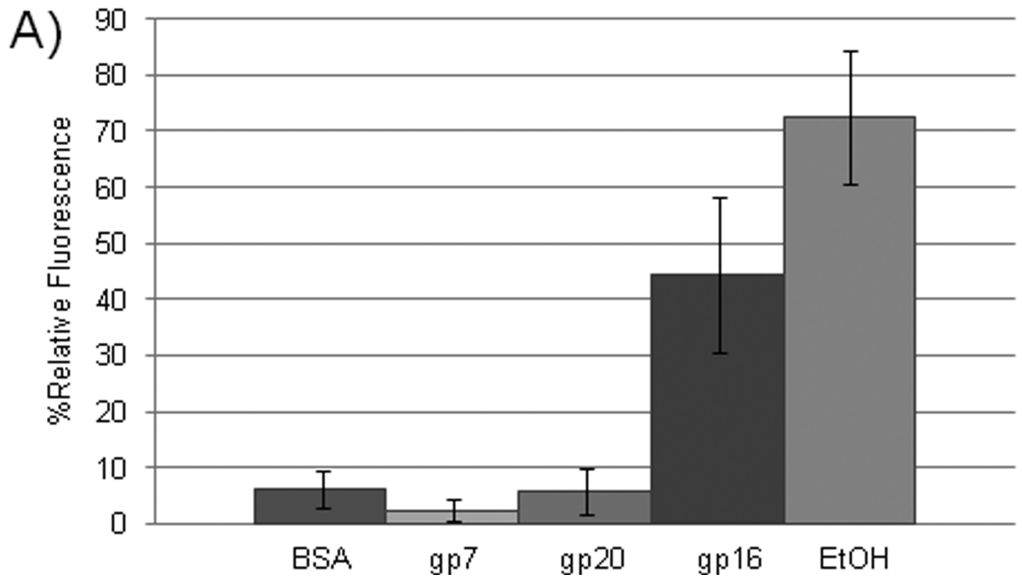
To determine the domain of gp16 required for membrane association, truncated constructs of gp16 were cloned into the pET46 expression vector and expressed in the *E. coli* expression host. All the constructs of gp16 were truncated at

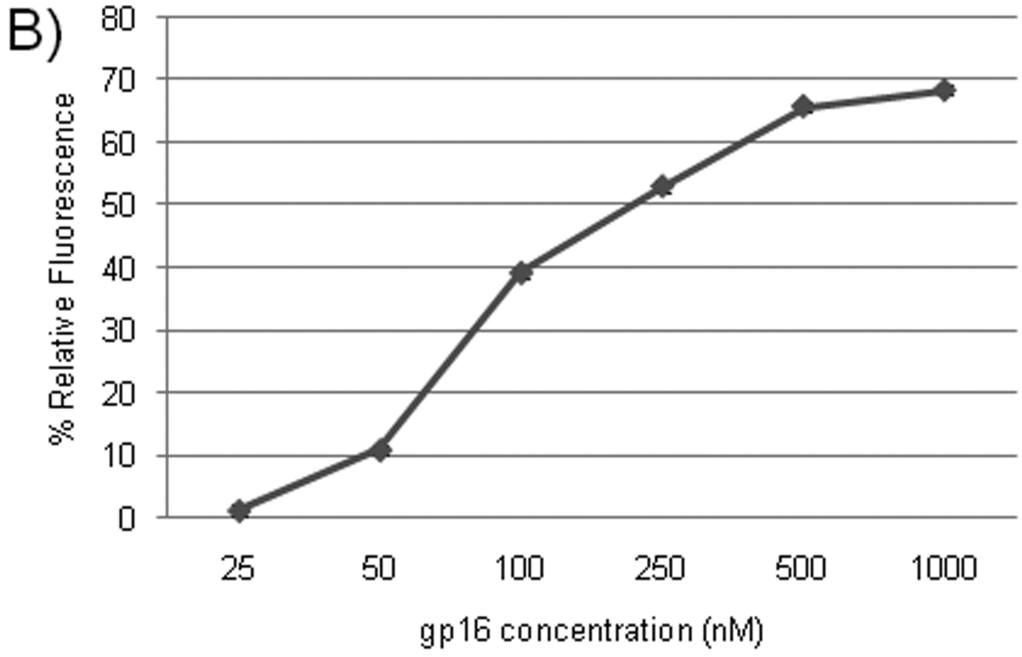
the C-terminal end. The purified full length (609 amino acids) and truncated constructs of gp16 were assayed for liposomal partitioning. Gp16 derivatives truncated from residues 476 - 609 partitioned into the liposomal fraction (Figure 2.3F), implying that the C-terminus of gp16 is not required for membrane association. In contrast, derivatives that are truncated from residues 301 - 609 partitioned in the soluble fraction instead of the liposomal fraction. This suggests that the domain between amino acid 301 to 475 is critical for membrane association of gp16 or is critical for proper folding of gp16 for membrane association to occur.

Do the pilot proteins form a membrane channel? The ability of the pilot proteins to form a transmembrane channel that could potentially transport the phage DNA across the host cytoplasmic membrane was assessed using liposomes with encapsulated fluorescent dye. The fluorescent dye calcein has minimal fluorescence when crowded inside liposomes, but release of the dye from the liposomes results in increased fluorescence.

Of the three pilot proteins, only gp16 was observed to promote leakage of the fluorescent dye from inside the liposomes as compared to the ethanol (positive) and BSA (negative) controls (Figure 2.4A). The increase in fluorescence was concentration-dependent (Figure 2.4B) and was observed within seconds after addition of gp16 to the liposomes (Figure 2.4C). To confirm the encapsulation of the fluorescent dye inside the liposomes, Triton X-100 was added to just the liposomes or to liposomes with gp7 after a few minutes of baseline fluorescence reading. The addition of Triton X-100 showed that calcein was indeed packaged inside the liposomes (Figure 2.4C). In addition, the presence of liposomes with encapsulated fluorescent dye was verified using transmission electron microscopy.

Figure 2.4: *Membrane-disrupting activity of the pilot proteins*. Samples were excited at 490nm and were read at an emission wavelength of 510 nm. Ethanol was used as the positive control, inducing almost complete lysis of the liposomes and BSA was used as the negative control. The % Relative Fluorescence was determined by calculating the ratio of the relative fluorescence values of the different samples to the relative fluorescence exhibited by 0.2% Triton X-100. (A) The pilot proteins, BSA and ethanol were added to liposomes with encapsulated fluorescent dye to determine their membrane-disrupting activity. Bar graph depicts the %Relative Fluorescence at the 2 min time point. (B) Increasing concentration of the gp16 protein were added to liposomes with encapsulated calcein dye to determine if dye leakage is concentration-dependent. Graph reflects the %Relative Fluorescence 2 min after the protein was added. (C) Triton X-100, gp16 and gp7 were added to the liposomes after 2 min of background fluorescence reading. After another 2 min, Triton X-100 was added to the liposomes with gp7 to demonstrate that the fluorescent dye was still inside the liposomes.


A)
%Relative Fluorescence
90
80
70
60
50
40
30
20
10
0
BSA
gp7
gp20
gp16
EtOH


B)
% Relative Fluorescence
80
70
60
50
40
30
20
10
0
25
50
100
250
500
1000
gp16 concentration (nM)

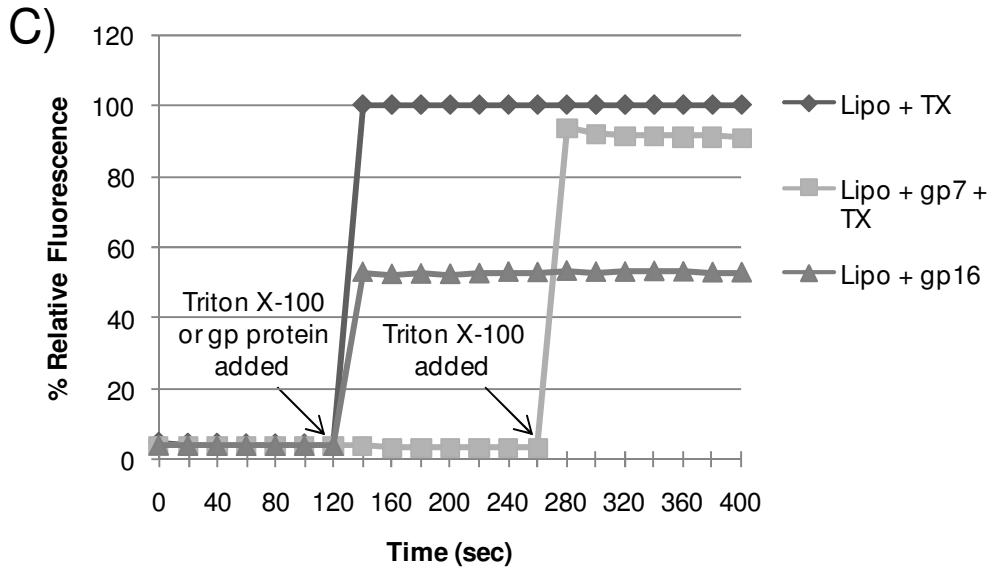


Figure 2.4: *Membrane-disrupting activity of the pilot proteins*, Continued.

Do pilot proteins facilitate DNA transport? Ability of the pilot proteins to facilitate DNA transport across membranes was investigated by following the uptake of ^{32}P-labeled DNA into liposomes. Addition of DNase I degraded any extra-liposomal DNA, allowing quantitation of DNA uptake into the liposomes.

No transport of DNA was observed in the absence of the pilot proteins. Transport of radiolabeled DNA was optimal when gp16 was present (Figure 2.5). Moreover, transport of the radiolabeled DNA only occurred in the presence of a membrane potential across the liposomal membrane. This suggests that the transport of phage P22 DNA across the cytoplasmic membrane of *Salmonella* is dependent on gp16 and the membrane potential of the host cell.



Figure 2.5: *Transport of DNA inside liposomes is gp16- and membrane potential-dependent.* Pilot proteins were incubated with the radiolabeled DNA prior to adding liposomes to the mixture. After 1 hr in room temperature, DNase I was added to digest all untransported DNA. Liposomes were disrupted with Triton X-100 + proteinase K and samples were run on a 5% native polyacrylamide gel. The gel was vacuum-dried and visualized with a phosphorimager. The artificial membrane potential was created by maintaining a 100mM KCl inside and a 20mM KCl outside the liposomes. The addition of valinomycin equilibrates the [K^+] inside and outside the liposomes generating a net negative charge inside the liposomes. Radiolabeled DNA was loaded in the first lane to mark the mobility of the DNA.

Discussion

The process of phage infection requires translocation of the phage DNA across the cytoplasmic membrane of the host cell. For most phage, the mechanism whereby phage DNA is transported across the inner membrane of the bacterial host has remained elusive. The process of phage DNA translocation into the host cytoplasm may occur by different mechanisms among different types of phages. Susskind, Botstein and Wright (173) provided convincing evidence that the P22 DNA is initially released in the periplasmic space and subsequently transported into the host cytoplasm. Our results indicate that for phage P22 the phage-encoded ejection protein gp16 is sufficient to catalyze the transport of DNA across the membrane *in vitro*. The membrane potential across the liposomal membrane is required to drive DNA transport into the liposome.

An alternative mechanism of phage DNA uptake was demonstrated by Molineux (119) who showed that the transport of phage T7 DNA across the cytoplasmic membrane of *E. coli* is enzyme-driven, not dependent on the pressure within the phage capsid. In contrast to T7, P22 DNA is inserted into the phage head by a headful packaging mechanism, and thus, the packaged DNA is circularly permuted. P22 is also an effective generalized transducing phage that can incorporate random fragments of the *Salmonella* host genome by a headful packaging mechanism. Because the initial end of the DNA fragments have different RNA polymerase binding sites, and because transport into liposomes can occur *in vitro* without additional enzymes, it is unlikely that P22 DNA is pulled into the host by a mechanism like that of T7. We tested the P22 ejection proteins for ATPase activity that might act as a motor to ratchet the DNA into the host cytoplasm but none of the proteins exhibited ATPase activity. On the other hand, in agreement with the *in vitro* results, inhibition of the membrane potential of *Salmonella* prevented uptake of the P22 DNA (detailed in Chapter 3). Thus, different bacteriophages use distinct mechanisms to transport their DNA into the bacterial host.

Johnson *et al*. recently published cryoelectron micrographs of the interior of the P22 capsid which show that the pilot proteins are localized at the portal where the tail meets the neck of the phage particle, suggesting that the pilot proteins interact prior to release from the phage head (74, 97). Thomas and Prevelige also found it difficult to recover pure gp16 from procapsids that contained both gp16 and gp20 (179). The two proteins cofractionated, presumably in a complex (179). In this study, the interactions between the three pilot proteins were confirmed *in vivo* by a series of pull down assays.

Targeting of the phage DNA to the outer leaflet of the cytoplasmic membrane is most likely a primary role of the gp16 protein since it was repeatedly found to be able to associate with the membrane fraction. Within the cytoplasmic membrane, gp16 may form a transport channel that mediates DNA uptake driven by the electrochemical gradient.

Deletion analysis suggested that a central segment of gp16 (amino acid 301 to 475) mediates membrane association. Bioinformatics reveals that this region has two amphipathic α-helices long enough to span the cytoplasmic membrane. The amphipathic α-helices from multiple gp16 proteins may interact to form a channel to transport a hydrophilic DNA molecule.

Thomas and Prevelige noted that gp16 self-polymerized into large structures in a concentration-dependent reversible reaction (179). This reaction was driven by heating and did not occur to any appreciable extent below 30^{o}. Although attempts to determine the morphology of these polymers were unsuccessful, the degree of scattering suggests that they were long rods (179). It is possible that this behavior is related to the protein's ability to form membrane pores.

Transport of radiolabeled DNA into liposomes only occurred in the presence of gp16. Although the addition of gp16 was sufficient to detect DNA transport *in vitro*, the presence of the other ejection proteins is essential during P22 infection (17, 70, 131). The presence of gp7 and gp20 may be required to protect the phage DNA in the periplasmic space. In addition, they may be required during the recircularization of the phage DNA once the linear double stranded DNA has entered the host cytoplasm (12).

The liposomal transport assay also revealed that P22 DNA transport is dependent on the membrane potential created across the liposomal bilayer. The *in vitro* requirement for a membrane potential strongly indicates that the membrane potential of the host cell might be critical for phage DNA transport across the cytoplasmic membrane. This agrees with previous studies which showed that phage T4 and T7 infection in *E. coli* requires a minimum threshold membrane potential (87, 95, 96).

Ejection proteins also facilitate the uptake of DNA from other phage into the cytoplasm. Like P22, the T7 tail is too short to span the *E. coli* cell envelope so a membrane channel is required to allow the phage DNA to enter the cytoplasm. It was proposed by Molineux *et al*. (86, 117, 119) that virion proteins are ejected into the cell, functionally endowing T7 with an extensible tail. The major functions of the ejected proteins are to form a channel across the cell envelope, including localized degradation of the cell wall (117, 119), and to translocate the leading end of the phage genome by what appears to be an enzyme-catalyzed reaction (52, 53, 87, 169). Molineux *et al*. showed that although T7 DNA is eventually pulled in by the host polymerase and then by the T7 polymerase into the *E. coli* cytoplasm in a transcription-dependent manner, the entry of the first few hundred basepairs of T7 DNA is transcription-independent and is controlled by a phage-encoded protein ejected from the phage head at the time of infection (53, 87, 169). The function of this protein is to regulate the entry of the phage DNA by clamping on the entering genome and limiting transcription-independent translocation to 850 bp, enough to allow binding of the *E. coli* RNA polymerase (87).

Although gp7, gp16, and gp20 of P22 are also ejected with the DNA during P22 infection, these proteins probably do not form an extensible tail like the internal proteins of phage T7 since gp16 acts *in trans* when *Salmonella* is coinfected with a gp16$^-$ P22 particle and a UV-irradiated P22 particle (67, 68). Since the coinfection produces a normal burst size, gp16 from the UV-irradiated phage must be able to diffuse inside the periplasmic space to bind the non-inactivated phage DNA and assist in its transport across the cytoplasmic membrane.

The *H* gene of phage lambda (λ) encodes a 90 kDa protein that is cleaved into a 78 kDa mature form called H* (83, 84). About 6 to 12 copies of the H* protein are present in the phage and seem to be ejected with λ DNA (141, 145). It has been proposed that H* may function as a “pilot protein”, which mediates transport of the DNA across the cytoplasmic membrane, but this has not been directly demonstrated (84).

In summary, these studies showed that the phage-encoded protein gp16 and the host membrane potential are necessary and sufficient for DNA translocation across the membrane *in vitro*.

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coli lactose permease. We would like to acknowledge Prof. Ian Booth for his valuable comments and suggestions.

Chapter 3. Membrane potential is required for transport of phage P22 DNA into *Salmonella enterica* sv. Typhimurium

Abstract

Phage P22 infects *Salmonella* Typhimurium. The short tail of P22 allows it to eject its DNA together with three pilot proteins into the periplasm of the host. The pilot proteins form a channel across the cytoplasmic membrane to transport the DNA into the host cytoplasm. To determine the energy requirements for P22 DNA transport, we tested the effect of inhibitors on DNA uptake. The addition of oligomycin or arsenate had no significant effect on transduction efficiency which suggests that ATP is not the direct energy source for DNA uptake from P22. In contrast, dinitrophenol significantly lowered the transduction efficiency implicating the proton motive force as the source for DNA uptake from P22. Nigericin, which specifically dissipates the proton gradient without disrupting the membrane potential, had no effect on transduction. Valinomycin with potassium chloride (KCl), which specifically dissipates the membrane potential without disrupting the proton gradient, reduced the transduction efficiency by 63%. The significant reduction in transduction in the presence of valinomycin plus KCl indicates that the membrane potential component of the proton motive force is the energy source for the transport of P22 DNA across the cytoplasmic membrane into the host cell.

Introduction

Phage P22 is a temperate, icosahedral, lambdoid phage used for generalized transduction of *Salmonella enterica* sv. Typhimurium. The short, noncontractile tail of

P22 facilitates the ejection of phage DNA together with three phage-encoded pilot proteins (gp7, gp16, and gp20) into the periplasmic space of the host cell (173). The pilot proteins both protect the DNA from degradative nucleases in the periplasmic space and form a channel across the cytoplasmic membrane that allows transport of the DNA into the cytoplasm of the host (Chapter 2).

Transport of a highly charged macromolecule like the DNA across an extremely hydrophobic bilayer like the cytoplasmic membrane of *Salmonella* Typhimurium requires energy (60, 77, 95, 96). It has been demonstrated in phage T4 and T7 that the energy for phage DNA transport into the cytoplasm of *E. coli* is provided by the membrane potential of the host cell (87, 95, 96). Infection of *E. coli* by phage T4 does not depend on the intracellular phosphate potential (78). The goal of this study was to determine the energy requirements for the transport of P22 DNA across the cytoplasmic membrane of *Salmonella* Typhimurium.

Materials and Methods

Bacterial strains. Strain MS1363 [*leuA414*(Am) Fels2$^-$] kindly provided by Prof. Mimi Susskind (USC) was used as the recipient strain for all P22 transductions. MST3663 [LT2 r$^-$m$^+$ (LT,SA) *leuA414*(Am) Fels$^-$ *endA* / pBR328 (Amp^R, Cam^R, Tet^R)] constructed by Lawes (100) was used as a donor for transduction. MST1120 [*srl::Tn10*] was used for the Tet efflux assay.

Phage strains. P22 HT105/1 *int-201*, a P22 high transducing phage carrying a mutation in the integrase gene, was used for transductions (110, 159). P22-64 [*virB*3 *sieA44 m44*] is a lytic strain of P22 that has a mutation in *sieA* in addition to

mutations in the DNA binding sites for the c2 repressor (20). The mutation in *sieA* prevents the exclusion of superinfecting DNA from occurring. This ensures that all host cells are lysed even in the unlikely event that P22 integration occurs. The mutation in the DNA binding sites for the c2 repressor also prevents lysogeny by preventing repression of genes required for lysis.

Chemicals. Leucyl-proline was purchased from Sigma. Fluorescamine, disodium tetraborate, and acetone were obtained from Fisher Scientific. JC-1 dye was purchased from Invitrogen.

Antibiotic concentrations. Chloramphenicol was used at a concentration of 20 µg/ml and Tetracycline was used at a concentration of 30 µg/ml.

P22 transduction. The P22 transduction protocol was based on the protocol of Maloy *et al*. (110). Briefly, P22 HT105/1 *int-201* was grown on MST3663 which harbors the chloramphenicol-resistant plasmid pBR328. The P22 lysate was titered and serially-diluted before use as donors in transducing the recipient strain MS1363. Aliquots of an overnight culture of MS1363 were incubated with different dilutions of the P22 lysate in a 37^{o}C water bath for 15 min for adsorption to occur. The mixtures were then plated on chloramphenicol plates, and the plates incubated overnight in a 37^{o}C incubator. The number of transductants was counted the next day for comparison of transduction efficiency between recipient cells that were treated with an uncoupler versus recipient cells that had no uncoupler added.

Uncouplers or inhibitors of proton motive force. Valinomycin was purchased from Sigma Aldrich. Oligomycin, arsenate, and nigericin were purchased from Fisher Scientific. When inhibitors prepared in 95% ethanol were used, dilutions

were made so that less than 1% ethanol was added to the cell culture and the same quantity of ethanol was added to the controls without inhibitors.

One-step growth curve. Cells from an overnight culture of MS1363 were subcultured 1:50 in LB broth and grown at 37°C to a cell concentration of ca. 10^8-10^9 cfu/ml. The specific chemical inhibitor was added to the cells and the cells were reincubated in a 30°C incubator with aeration for 30 min to allow the inhibitor to take effect. A 100 µl aliquot of the cells was taken to check for cell viability prior to mixing a P22-64 lysate with the cells at an MOI of 1. The cells were incubated at 37°C with aeration for 3 hr to allow for cell lysis while taking 100 µl aliquots at the 0, 20, 30, 60, 90, 120, 150, and 180 min time points. Aliquots were vortexed in a tube with 100 µl of chloroform and the tubes were placed on ice until the supernatant was titered.

Titering of P22 particles. Evans Blue Uranine (EBU) plates were layered with 4 ml of melted soft agar containing 100 µl of MS1363 host cells. The collected 100 µl aliquots from the one-step growth curve were vortexed and centrifuged at 14K rpm for 3 min in a tabletop microcentrifuge. The supernatant was serially-diluted to 10^{-8} and 10 µl spots of the serial dilutions were applied to an EBU plate with a soft agar layer of MS1363 host cells. The spots were allowed to dry before the EBU plates were incubated overnight in a 37°C incubator. The number of plaques was counted the next day to determine the number of unadsorbed phage particles at each time point.

ATPase assay. The ATPase assay was performed as described in Hopfe and Henrich (69) and Henkel *et al.* (64). Briefly, the assay was performed in microtiter plates by incubating 10 µg of purified protein in 20 µl of buffer A (120 mM NaCl, 5 mM

KCl, 20 mM Tris-HCl, pH 7.5) with 5 mM ATP and 5 mM $MgCl_2$ for 30 to 60 min at 37°C. Hydrolysis of ATP was terminated by adding 200 µl of malachite green reagent (5.72% [wt/vol] ammonium molybdate in 6 N HCl, 2.32% [wt/vol] polyvinyl alcohol, 0.0812% [wt/vol] malachite green, and distilled water at a ratio of 1:1:2:2). The relative absorbance of the samples in relation to a blank was measured at 620 nm. Inorganic phosphate (in concentrations varying from 1 to 20 nmol) was used as a standard.

Making wild-type Salmonella Typhimurium cells permeable to valinomycin. The protocol for making cells permeable to valinomycin was based on the procedure described in Labedan and Goldberg (95) and Labedan *et al*. (96). Briefly, *Salmonella* cells were grown at 37°C to 2 x 10^9 cells/ml in 30 ml of LB broth and then washed twice at room temperature with 30 ml of 0.1 M Tris buffer, pH 8.0. Cells were resuspended in 6 ml of 1 M Tris pH 8.0 and placed in 37°C water bath for 8 min. Cells were exposed to a final concentration of 10 mM EDTA for another 8 min in 37°C and 22 min in room temperature. Cells were washed twice with 30 ml of LB broth to remove EDTA and then resuspended in 24 ml of LB broth. 4 ml aliquots of the permeabilized cells were either treated with inhibitors or left untreated. When treated, valinomycin was added to a final concentration of 10 nM.

Results

The P22 pilot proteins do not exhibit ATPase activity. The energy source for DNA uptake from phage P22 has not previously been determined. One suggestion is that the P22 pilot proteins hydrolyse ATP to supply the energy for DNA transport.

The pilot proteins were tested for ATPase activity to determine if they are capable of breaking down ATP and using the energy from ATP hydrolysis to transport the phage DNA across the cytoplasmic membrane. None of the pilot proteins tested positive for ATPase activity (Figure 3.1). The malachite green ATPase assay detects the release of inorganic phosphates from the hydrolysis of ATP.

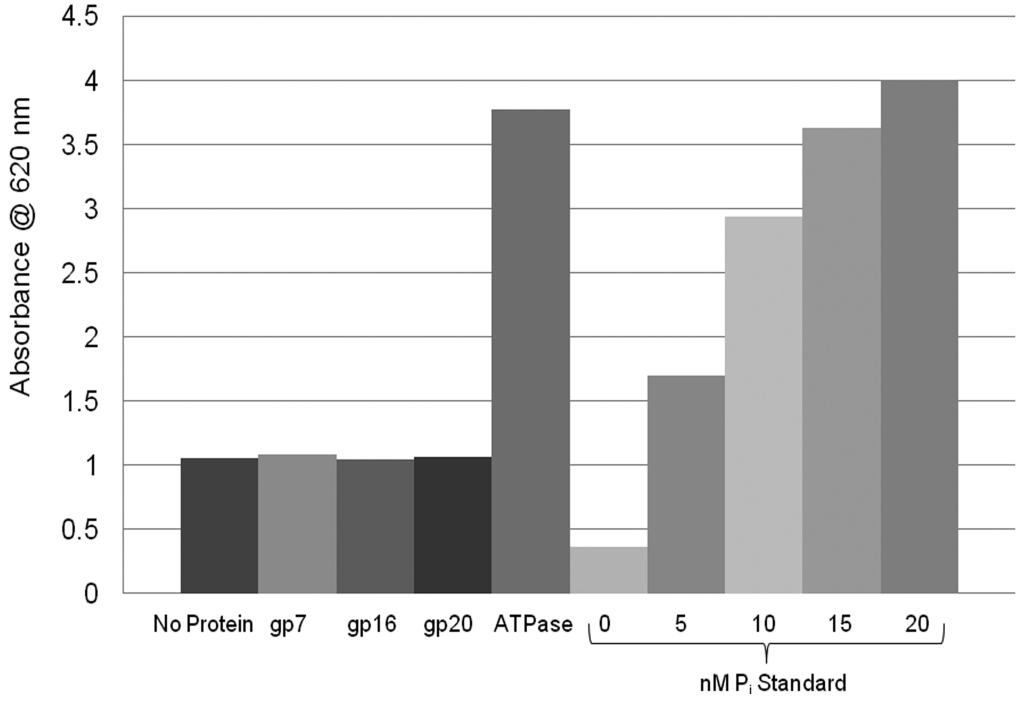


Figure 3.1: *Pilot proteins do not have ATPase activity*. 620 nm absorbance readings of the inorganic phosphate (P_i) standards show the validity of the assay. An ATPase with an activity of 0.1 µmol/min/ml was used as a positive control. 620 nm absorbance readings of the pilot proteins compared to the "no protein" control (Buffer A + ATP + $MgCl_2$) show that the pilot proteins have no ATPase activity. A total of 10.0 µg of each pilot protein was added in each reaction. Only the 30 min incubation readings are shown but readings from the 1 hr incubation are similar to the readings of the 30 min incubation.

ATP hydrolysis by the host is not involved in the uptake of P22 DNA. The hydrolysis of ATP to ADP or AMP by the host could also power the transport of P22 DNA across the cytoplasmic membrane. To test the involvement of ATP in the uptake

of P22 DNA, either oligomycin or arsenate was added to the recipient cell culture prior to P22 transduction. Oligomycin disrupts ATP synthesis by inhibiting the F_OF_1 synthase while arsenate prevents ATP synthesis by competing with inorganic phosphates in the synthesis of ATP from inorganic phosphate plus ADP or AMP (78). Neither oligomycin nor arsenate was found to affect P22 transduction (Table 3.1). The depletion of intracellular ATP by either disrupting the F_OF_1 synthase or by preventing the synthesis of ATP from ADP or AMP did not lower the number of transductants compared to the untreated sample. This suggests that ATP is not directly involved in the uptake of phage P22 DNA into the host cell. The activity of arsenate and oligomycin to deplete ATP was tested on the transport of the dipeptide leucyl-proline into wild-type *Salmonella* cells (Figure 3.2).

Table 3.1: Effect of inhibitors on P22 transduction

Inhibitors[a]	Transduction Efficiency (%)[b]	Viability cfu/ml)
No inhibitor	100[c]	7.0×10^8
2 mM DNP	0.2	3.0×10^8
100 µM Oligomycin	83.1	6.7×10^8
3 mM Arsenate	126.9	2.0×10^9
5 nM Nigericin	176.5	2.1×10^9
5 nM Nigericin + 3 mM Arsenate	85.5	1.6×10^9

Table 3.1: Effect of inhibitors on P22 transduction, Continued.

Inhibitors[a]	Transduction Efficiency (%)[b]	Viability cfu/ml)
No inhibitor[d]	100[c]	1.7 X 10^8
100 mM KCl[d]	95.4	2.5 X 10^8
10 nM Valinomycin[d]	66.5	0.9 X 10^8
10 nM Valinomycin + 100 mM KCl[d]	37.3	0.9 X 10^8
3 mM Arsenate[d]	105.8	2.5 X 10^8
10 nM Valinomycin + 100 mM KCl + 3 mM Arsenate[d]	35.9	0.7 X 10^8

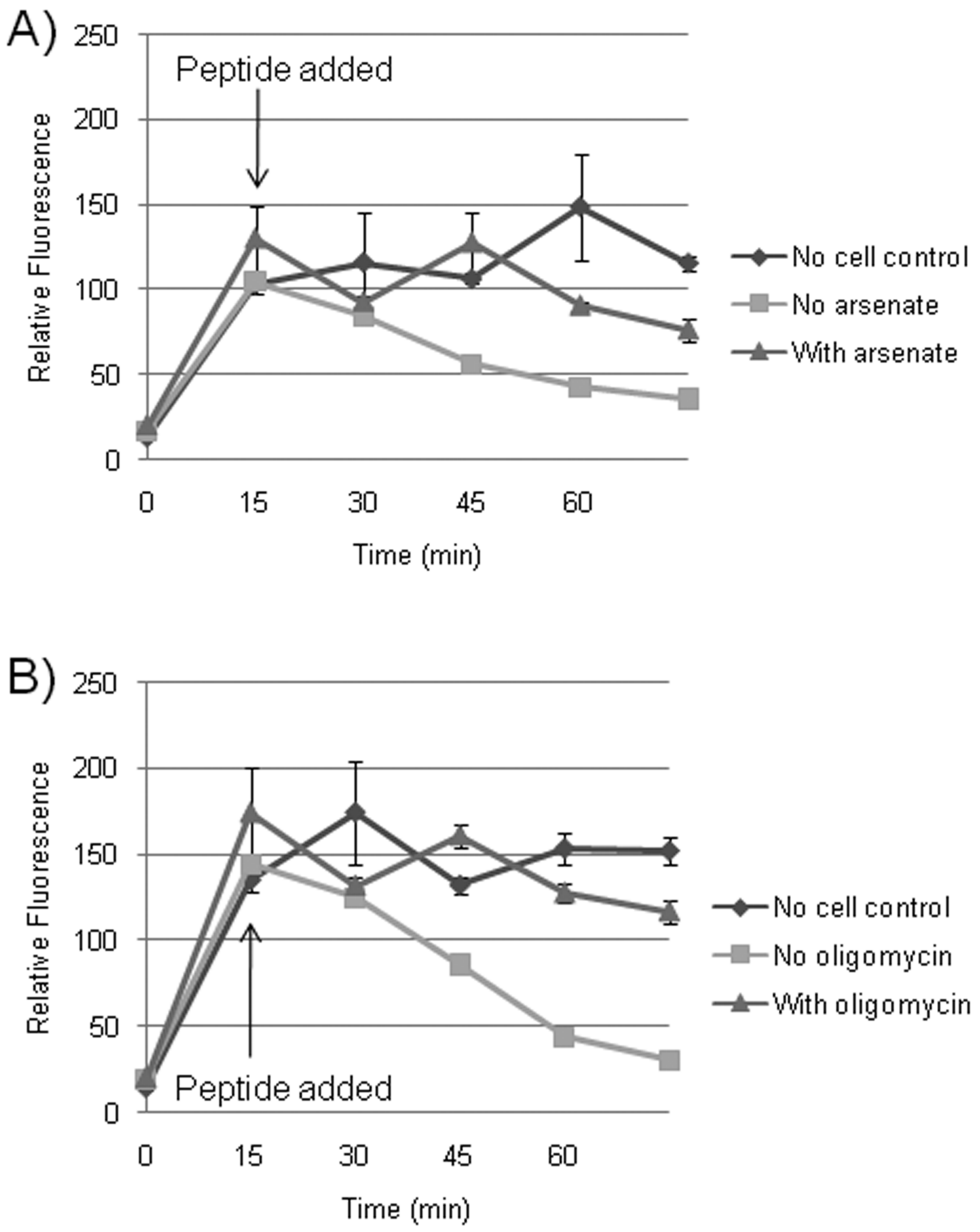
[a]The concentration of inhibitors used was optimized to have very little effect on cell viability to ensure that the effect on transduction efficiency by the inhibitor is not simply due to cell toxicity. The concentration range of the different inhibitors used in this study was based on previous experiments conducted on phage T4 infection in *E. coli* (96).

[b]Data shown reflect average values of at least three independent experiments.

[c]The number of Cm^R transductants without any inhibitors was arbitrarily assigned 100% transduction efficiency to normalize all transduction results in the presence of the different inhibitors.

[d]Host cells were permeabilized to allow valinomycin to penetrate cells.

Figure 3.2: *Arsenate and oligomycin inhibit the ATP-dependent transport of leucyl-proline into wild-type Salmonella.* Bacteria were either treated with 3 mM arsenate (A), 100 µM oligomycin (B) or not treated with any inhibitor prior to the addition of 10 nmol of the dipeptide. The disappearance of the dipeptide from the medium was assayed using fluorescamine, the method being based on Payne and Nisbet (128). Samples (0.5 ml) of the incubation medium were removed periodically, filtered immediately to remove microorganisms, 0.05 ml filtrate was added to 2.5 ml 0.1 M disodium tetraborate (pH 6.2), and 0.5 ml fresh fluorescamine solution (0.15 mg/ml in acetone) was added with vortex mixing. After 5 min of incubation at room temperature in the dark, peptide concentration was determined from the fluorescence of the solution (excitation 390 nm; emission 485 nm).



The host cell provides the energy for phage P22 DNA transport. If the pilot proteins cannot provide the energy of transport in the form of ATP hydrolysis, then the transport energy is most likely obtained directly from the host cell. There are two possible sources of transport energy that the host cell can provide. The host cell can potentially use ATP either synthesized via oxidative phosphorylation or via

substrate-level phosphorylation. Alternatively, the host cell can directly use the proton motive force across its cytoplasmic membrane to drive the DNA transport.

Evidence that the transport energy is provided by the host was obtained by performing a transduction assay in the presence or absence of 2 mM dinitrophenol (DNP) (Table 3.1). DNP is an ionophore that specifically transports protons across membranes to dissipate the proton motive force. Transduction was performed by infecting wild-type *Salmonella* Typhimurium with a high transducing P22 particle that is incapable of integrating into the *Salmonella* genome by virtue of a mutation in the integrase (*int*) gene. The high transducing P22 particles were propagated in a *Salmonella* strain which contains multiple copies of the pBR328 plasmid that confers chloramphenicol resistance (Cm^R). pBR328 plasmids were packaged as concatemeric DNA (189) and the P22 transducing particles that have packaged the pBR328 plasmids were then used to transduce wild-type *Salmonella* Typhimurium that were either pre-incubated with DNP or untreated. Plating the transductants on chloramphenicol plates selected for recipient cells that had inherited the pBR328 plasmid. There were over 500 Cm^R transductants (arbitrarily designated as 100% transduction efficiency) when no DNP was added to the recipient cells. In contrast to the untreated cells, the number of Cm^R transductants (0.2% transduction efficiency) when a final concentration of 2 mM DNP was added to the cell culture was very low (Table 3.1). The decrease in the number of transductants was not due to loss of cell viability since the number of viable DNP-treated cells (3×10^8 cfu/ml) was not substantially different from the number of viable non-treated cells (7×10^8 cfu/ml). The inhibitory effect of DNP on the uptake of phage DNA not only confirms that the host is the source of energy for phage DNA transport but also indicates that the

proton motive force is the major contributing source of energy powering the transport of DNA.

DNP does not affect P22 adsorption. The inhibitory effect of DNP on the uptake of phage P22 DNA is not due to the inability of P22 transducing particles to adsorb to the host cell. The one-step growth curve of P22 particles infecting wild-type *Salmonella* Typhimurium shows that P22 adsorption proceeds normally even in the presence of 10 mM DNP (Figure 3.3). This data suggests that the inhibitory effect of 2 mM DNP in preventing transduction (Table 3.1) occurs after adsorption of P22 has occurred.

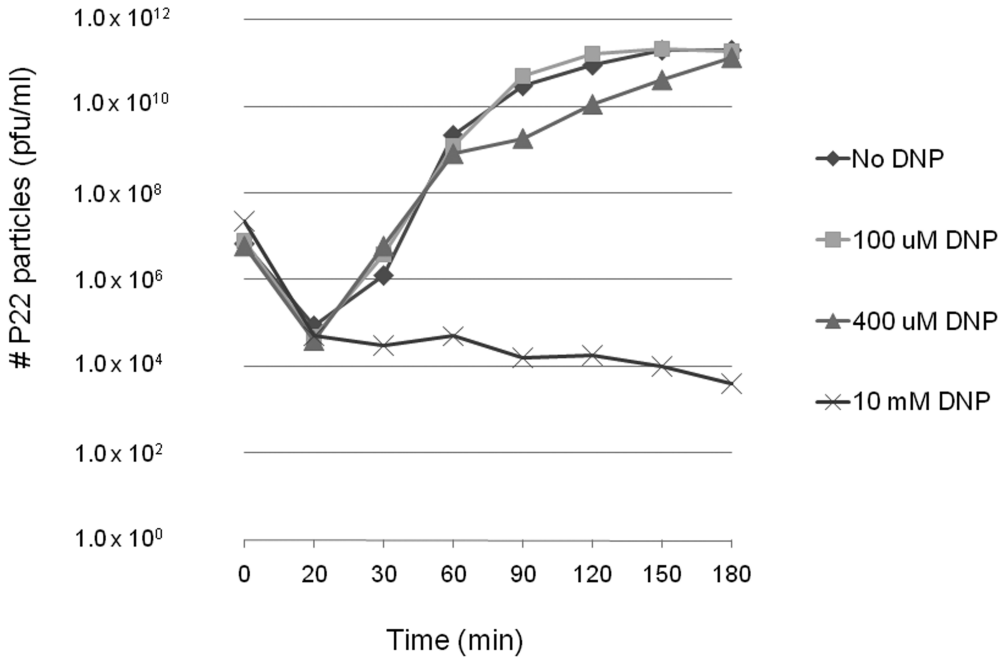


Figure 3.3: *DNP does not affect P22 adsorption*. The decrease in the number of phage particles in the supernatant from the 0 time point to the 20 min time point corresponds to the irreversible adsorption of phage particles to host cells.

Concentrations of DNP tested did not substantially reduce the viability of the cells before the addition of the lytic strain of P22 (Table 3.2). This suggests that the inhibitory effect of 2 mM DNP on transduction is not due to cell toxicity. Furthermore, 10 mM DNP made the recipient cells resistant to P22 infection as evidenced by the lack of decrease in cell viability after 3 hrs of incubation with the lytic strain of P22. This is possibly because 10 mM DNP prevented the uptake of P22 DNA which made the host cells resistant to P22 infection.

Table 3.2: Cell viability before P22 addition and 3 hrs after P22 addition

P22 addition	DNP Concentration			
	None	100 µM	400 µM	10 mM
No treatment[a]	5 x 10^{9}[b]	3 x 10^{9}	4 x 10^{9}	3 x 10^{9}
0 hr before treatment	7 x 10^{8}	8 x 10^{8}	9 x 10^{8}	4 x 10^{8}
3 hr after treatment	2 x 10^{4}	2 x 10^{4}	7 x 10^{3}	3 x 10^{8}

[a]Cell were grown without P22-64 for another 3 hr after addition of DNP

[b]Cell viability in cfu/ml

P22 DNA transport is dependent on the membrane potential of the host. The proton motive force across the cytoplasmic membrane is composed of two components: the proton gradient and the membrane potential. To determine which component is the energy source for transport of phage P22 DNA, either valinomycin

or nigericin were added to the recipient cells before P22 transduction was performed. Valinomycin is an ionophore that specifically transports potassium across membranes, thereby dissipating the membrane potential without affecting the proton gradient. Nigericin is an ionophore that exchanges potassium ions for protons and thereby dissipates the proton gradient but not the membrane potential.

Valinomycin but not nigericin has an effect on the uptake of P22 DNA (Table 3.1). The transduction efficiency when the cells were treated with nigericin is not significantly different from the transduction efficiency of the untreated sample. On the other hand, the cells treated with valinomycin gave a significantly lower transduction efficiency compared to the untreated sample. The concentration of inhibitors used in the assay had very minimal effect on the viability of the cells. These results suggest that the membrane potential component of the proton motive force is responsible for providing the energy for the successful transport of phage P22 DNA across the cytoplasmic membrane of the host cell.

The effect of valinomycin on the membrane potential was confirmed by using the cationic dye JC-1 (Molecular Probes). The membrane potential component of the PMF can be measured in cells using the JC-1 dye (10, 75). JC-1 indicates membrane depolarization by shifting its fluorescence emission from red (~590 nm) to green (~530 nm) after excitation at 485 nm (75). Valinomycin but not nigericin depolarizes the membrane potential across *Salmonella* cells (Figure 3.4). Cells treated with nigericin show the same fluorescence as wild-type cells.

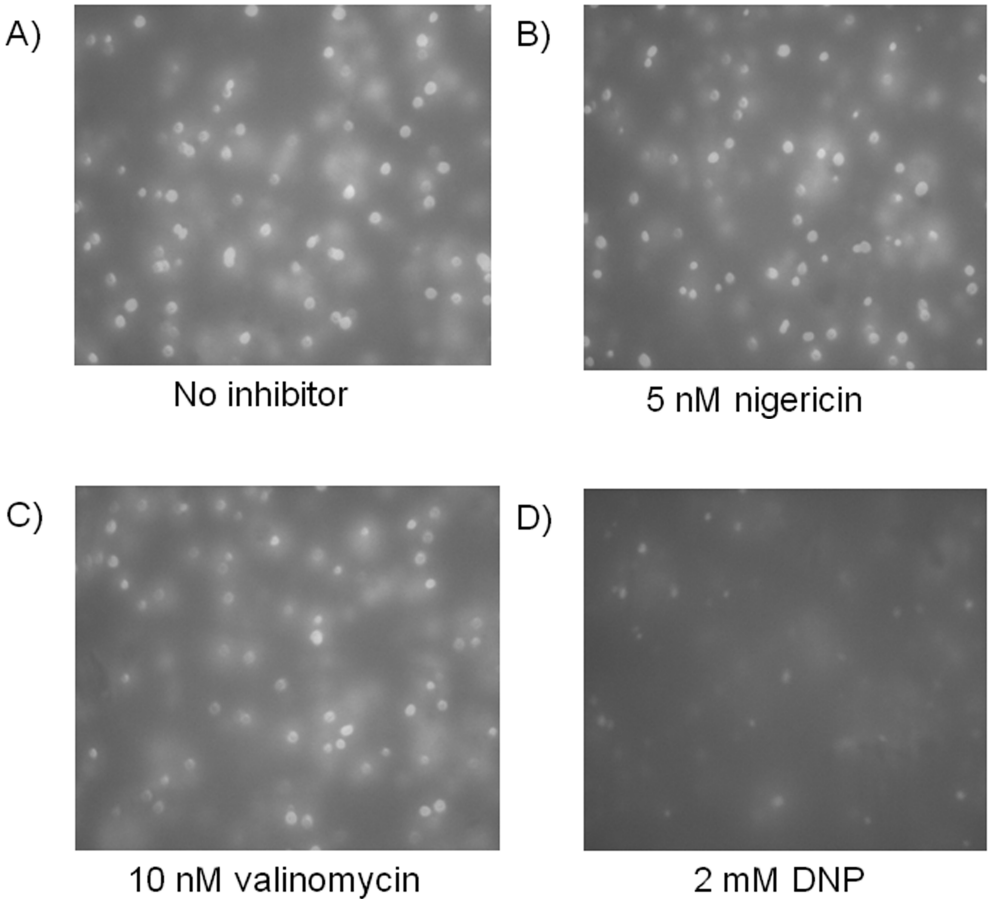


Figure 3.4: *Valinomycin but not nigericin depolarizes the membrane potential across the Salmonella membrane*. The cationic dye JC-1 (Molecular Probe) was used to indicate membrane depolarization by shifting its fluorescence emission from red (~590 nm) to green (~530 nm) after excitation at 485 nm (75). Cells were permeabilized to allow valinomycin to penetrate the cell membrane as described in materials and methods. Permeabilized cells were either treated with 5 nM nigericin (B), 10 nM valinomycin (C), 2 mM DNP (D), or not treated with any inhibitor (A). JC-1 (2 µl of 5 mg/ml) was added to all samples and the samples were incubated for 30 min at room temperature. Slides were prepared and the fluorescent bacteria were examined using a fluorescence microscope at an excitation wavelength of 485 nm.

The effect of nigericin on the pH gradient was tested by assaying the efflux of tetracycline from inside *Salmonella* cells. The efflux of tetracycline is dependent on the pH gradient and not on the membrane potential of the cell (79, 188).The Tet^R strain of *Salmonella* (MST 1120) had difficulty growing on a Tet (30 µg/ml) plate in the

presence of 5 nM nigericin, suggesting that nigericin facilitates tetracycline accumulation inside the cells (Table 3.3). Although 5 nM nigericin did not have any effect on cell viability, cells treated with 5 nM nigericin had difficulty pumping out tetracycline from inside the cells.

Table 3.3: Effect of nigericin on Tetracycline efflux

Inhibitors	Efflux Efficiency (%)[b]	Viability (cfu/ml)
No inhibitor	100[c]	1.6×10^6
5 nM Nigericin[a]	3.4	1.3×10^6

[a]The concentration of nigericin used was optimized to limit effects on cell viability to ensure that the effect on efflux efficiency by the inhibitor is not simply due to cell toxicity. The concentration range of the different inhibitors used in this study was based on previous experiments conducted on phage T4 infection in *E. coli* (96).

[b]Efflux efficiency was performed on LB plates with Tet (30 µg/ml). Data shown reflect average values of at least three isolated experiments.

[c]The number of colony forming units per ml of culture without nigericin was arbitrarily assigned 100% efflux efficiency to normalize results in the presence of the inhibitor.

Discussion

The transport of charged molecules across the phospholipid bilayer of the cytoplasmic membrane requires energy. The possible sources of this required energy are via ATP hydrolysis or in the form of the established proton motive force across the cytoplasmic membrane.

Before examining the host as the source of energy for the transport of phage DNA across the cytoplasmic membrane, the individual pilot proteins were tested for signs of ATPase activity. The malachite green ATPase assay showed that none of the pilot proteins demonstrated the ability to hydrolyze ATP.

Moreover, depleting ATP in the host using oligomycin or arsenate did not lower transduction efficiency. This suggests that the transport of phage DNA across the cytoplasmic membrane is not driven by ATP.

Alternatively, transport could be driven by PMF. The addition of 2 mM dinitrophenol (DNP) lowered the transduction efficiency to 0.2% of untreated cells. This indicates that the proton motive force is the major energy source driving the uptake of phage DNA.

The inhibitory effect of DNP on the uptake of phage P22 DNA is not due to the inability of P22 transducing particle to adsorb to the host cell. The one-step growth curve of P22 particles infecting wild-type *Salmonella* Typhimurium shows that P22 adsorption proceeds normally even in the presence of 10 mM DNP. The data shown is consistent with the fact that the inhibition of transduction by DNP occurs after adsorption of P22 to the host.

To determine which component of the proton motive force is required for transport of P22 DNA across the cytoplasmic membrane, the effects of valinomycin and nigericin on P22 transduction were investigated. Nigericin, an ionophore that specifically dissipates the proton gradient without disrupting the membrane potential, did not have an effect on transduction. In contrast, valinomycin (+ KCl) which is an ionophore that dissipates the membrane potential without disrupting the proton

gradient, lowered the transduction efficiency by 63%. Taken together, these results suggest that the membrane potential provides the energy for the uptake of P22 DNA across the cytoplasmic membrane of the host cell.

Other phages also use the membrane potential of the host to power the translocation of the phage DNA across the cytoplasmic membrane. Labedan *et al*. (95) showed that the membrane potential, not the proton gradient, is responsible for the entry of the phage T4 DNA into the cytoplasm of *E. coli*. This was also shown to be the case for phage T7 (87). This could be a common feature of DNA transport from phages.

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Chapter 4. Host factors for the uptake of phage P22 DNA into the cytoplasm of *Salmonella enterica* sv. Typhimurium

Abstract

The mechanism of phage P22 DNA transport across the cytoplasmic membrane of *Salmonella* Typhimurium is not known. P22 DNA is initially released into the periplasmic space and subsequently transported into the cytoplasm of the host. Host proteins may be involved in translocating the phage DNA across the cytoplasmic membrane. It is possible that a pre-existing host factor is sequestered by the P22 pilot proteins (gp7, gp16, and gp20) to channel the phage DNA across the cytoplasmic membrane. Transposon and chemical mutagenesis were performed on wild-type *Salmonella* Typhimurium to screen for mutants resistant to P22 infection. P22-resistant colonies were selected on Evans Blue Uranine plates supplemented with a virulent mutant of P22. Because LPS mutants have been extensively characterized, $P22^R$ mutants that had a mutation in genes involved in LPS biosynthesis were eliminated using phage Felix-O1 and phage Ffm. The remaining $P22^R$ mutants were grouped using a one-step growth curve to determine which stage of the P22 infection process is inhibited. A transduction assay was performed to identify $P22^R$ mutants that have a specific mutation in transduction. Suppressor analysis was also employed as an alternative genetic tool in screening for factors involved in phage DNA transport. Most of the $P22^R$ mutants created by transposon and chemical mutagenesis had a mutation in genes involved in LPS biosynthesis. Nitrosoguanidine mutagenesis generated five $P22^R$ mutants with normal adsorption kinetics and one of these mutants had a deficiency in transduction. Suppressor

analysis failed to isolate host-encoded or phage-encoded factors involved in phage DNA transport. Biochemical approaches were explored to complement the genetic studies. Purified 6xHis-tagged derivatives of the P22 pilot proteins were used to “fish out” potential host factors by co-elution. The use of cross-linkers and Virus Overlay Protein Blot Assay (VOPBA) were investigated to narrow down the pool of potential host factors. Biochemical approaches revealed a 34 kDa host protein that interacted strongly with the gp16 protein. The VOPBA approach proved useful in isolating a 40 kDa host membrane protein that specifically interacted with 6xHis-gp16 and 6xHis-gp7. The 34 kDa host protein isolated *in vivo* and the 40 kDa host membrane protein isolated by VOPBA are potential host factors responsible for the transport of phage P22 DNA across the cytoplasmic membrane of *Salmonella*. The unsuccessful isolation of a host factor by transposon mutagenesis and the observation that the only $P22^R$ mutants obtained had a partial deficiency in transduction strongly support the hypothesis that the host factors are encoded by essential genes.

Introduction

Some phages eject their DNA into the periplasm then subsequently translocate their DNA across the cytoplasmic membrane of their host cell. For example, after phage lambda adheres to the surface of *E. coli*, the phage-encoded H protein is ejected with the lambda DNA into the periplasmic space (141, 145). It has been proposed that the H protein may function as a pilot protein which mediates transport of DNA across the cytoplasmic membrane (84).

Host proteins may also be involved in the uptake of lambda DNA. The *E. coli pel* gene (penetration of lambda) seems to play a role in lambda DNA uptake,

because pel mutants show normal adsorption of phage lambda but only 2 to 10 percent of the infected cells yield a normal burst of progeny phage or form lysogens (156). The *pel*$^-$ mutations map in the *manXYZ* operon which encodes protein II-P of the mannose phosphostransferase system (42, 45, 127). The *pel* mutations are alleles of *manY* (5) which encodes the mannose permease.

Mutants of phage lambda were isolated which could infect *pel* mutants (157). The mutants were designated lambda *hp* for host range of *pel*. All *hp* alleles mapped in the lambda *H* gene. Since the lambda *hp* mutations mapped within the *H* gene, the H pilot protein may itself interact with *manY* (*ptsM*). Thus, the mannose permease may be a host factor used by phage lambda to translocate its DNA from the periplasmic space into the cytoplasm of the host. In addition to phage lambda, phages 434, 82, and N4 are also affected by *pel* mutations (88), suggesting that these phages also use the mannose permease to translocate their phage DNA into their host cytoplasm even though they use a different outer membrane receptor than phage lambda to adhere to their specific host.

The Ff (F-factor specific filamentous) coliphages f1, fd, and M13 also seem to use a host factor to transport their DNA across the cytoplasmic membrane of their host. These phages are restricted in host range to F$^+$ *E. coli*. They use the tip of the *E. coli* F pilus as a receptor to adsorb to the surface of the host (22, 151). After interaction with the phage, the pilus is withdrawn into the bacterial cell surface, pulling the phage into close association with the outer membrane. The Ff phages have 3 to 5 copies of the gene 3 protein (gp3) located at one end of the phage coat (56, 136) which interacts with the pilus tip and also functions in ejecting the phage DNA into the

periplasmic space possibly by forming a pore as a trimer on the outer membrane of the host bacterium.

TolA is an integral membrane protein found in *E. coli* that is comprised of 3 domains. Domain I targets and anchors TolA in the cytoplasmic membrane. Domains II and III are periplasmically located. Domain II is a contiguous alpha-helical stretch while domain III encodes the TolA function. The N-terminus of the gp3 protein of the Ff phage binds to domain III of TolA *in vitro* (11). It has been proposed that domain II of TolA links domains I and III together so that domain III is able to interact with phage proteins at the outer membrane while remaining tethered to the cytoplasmic membrane (106). Although domain III of TolA was shown to interact with the gp3 protein of the Ff phage, it is still not known whether the phage DNA actually enters the periplasm through this interaction.

The purpose of the present study is to investigate the potential involvement of host proteins in the transport of phage P22 DNA across the cytoplasmic membrane of *Salmonella* Typhimurium. In addition to the phage-encoded pilot proteins, host factors might also contribute to the translocation of the phage DNA. The pilot proteins might interact with and modify a pre-existing host-encoded channel protein to facilitate phage DNA translocation.

P22 is a temperate, icosahedral, “lambdoid” phage that infects *Salmonella enterica* sv.Typhimurium. The mechanism of how the phage P22 DNA crosses the cytoplasmic membrane of the host is still not known. Like phage lambda, it is possible that P22 is also using a host protein to translocate its DNA across the cytoplasmic

membrane of *Salmonella*. This chapter presents both genetic and biochemical approaches to identify host factors potentially involved in P22 uptake into *Salmonella.*

Two genetic approaches for isolating mutants in the host factor involved in P22 uptake were used: direct genetic analysis and suppressor analysis. The direct genetic analysis involved randomly mutagenizing wild-type *Salmonella* Typhimurium and then selecting for $P22^R$ colonies. The rationale for selecting for $P22^R$ colonies is that a mutation in the gene that codes for a host factor required for phage DNA uptake should render the host resistant to P22 infection. The suppressor genetic analysis involved mutagenesis of a P22 *sieA*$^+$ lysogen strain of *Salmonella* Typhimurium. The superinfection exclusion (SieA) protein encoded by the P22 prophage renders the host bacterium resistant to infection by superinfecting P22 particles (173). The P22 *sieA*$^+$ lysogen was randomly mutagenized and mutants were screened for P22 sensitivity. Sensitivity of the mutagenized P22 *sieA*$^+$ lysogen to an incoming P22 DNA would suggest that a host gene whose gene product is directly involved in the mechanism of superinfection exclusion has been mutated such that the SieA protein could no longer exclude the superinfecting P22 DNA. The superinfecting P22 DNA was also mutagenized as part of the suppressor analysis in an effort to determine whether the factors responsible for DNA uptake are phage-encoded.

The techniques used for the direct and suppressor genetic analyses were transposon mutagenesis and chemical mutagenesis. The three transposons used to create $P22^R$ mutants were Tn*phoA*, Tn*5,* and T-POP. Once introduced into the recipient cell, the transposon can randomly insert into the genome of the recipient cell (Figure 4.1). All the transposons used have an antibiotic resistance marker that

facilitates the selection of the transposon insertion mutants. One difference between the three transposons is the way they were packaged and delivered into the recipient cell. Tn*phoA* which has its own copy of the transposase gene was packaged using a derivative of P22 called MudP22 and the transducing particles containing the Tn*phoA* transposon were used to introduce Tn*phoA* into the recipient cell. The mini-Tn*5* transposon which carries the Kanamycin resistance cassette was introduced into the recipient cell by electroporation of the pRL27 plasmid which contains both the mini-Tn*5* transposon and the transposase. The *ori* R6K origin of replication on the pRL27 vector restricts its replication to host cells that can produce the pi (π) protein (encoded by the *pir* genes). Introduction of the Tn*5*-RL27 plasmid into a *pir* ⁻ recipient cell followed by selection on a Kanamycin plate results in the selection of transposon mutants with a subsequent loss of the transposase.

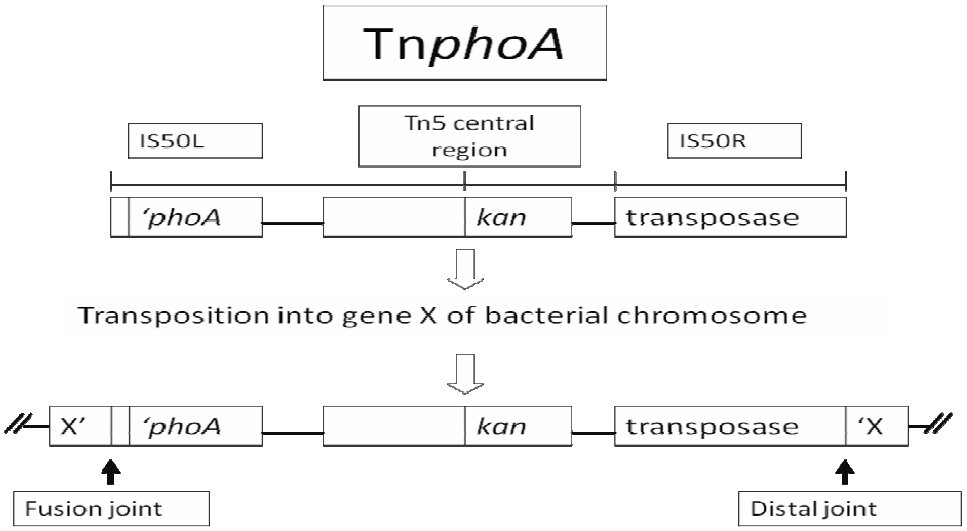


Figure 4.1: *The Tn*phoA *transposon*. The Tn*phoA* transposon can hop into any gene of the bacterial chromosome (designated by gene X) by the action of the transposase enzyme. The Tn*5* transposon which contains the Kanamycin resistance cassette sits in the central region of Tn*phoA*. The transposase enzyme acts upon the left and right insertion elements (IS50L and IS50R) to catalyze the "cut and paste" mechanism of transposition.

The chemical mutagens employed in the direct and suppressor genetic analyses were ethylethane sulfonate or diethylsulfate (DES), and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG). DES and MNNG are both alkylating agents that add an alkyl group to the hydrogen-bonding oxygens of G and T. The alkylation impairs the normal hydrogen bonding of the bases, causing mispairing of G with T. DES mostly produces G-C → T-A transversions while MNNG produces multiple G-C → A-T transitions (111).

Phage DNA mutagenesis was performed either by using hydroxylamine (HA) or by propagating the phage in a mutator (*mutD*) strain of *Salmonella* Typhimurium. Hydroxylamine is also an alkylating agent that reacts specifically with C, converting it to a modified base that pairs with A instead of G producing G-C → A-T transitions (111). The *mutD* strain of *Salmonella* Typhimurium has a mutation in the *dnaQ* gene which is required for the proofreading function of DNA polymerase III. The resulting defect in DNA repair increases the frequency of mutagenesis. Thus, the mutation frequency of a phage that is propagated in a mutator strain is greatly enhanced (111).

The biochemical approach sought to co-purify any host proteins that may be involved in phage P22 DNA transport. This approach was based on the hypothesis that the pilot proteins, especially the membrane-associating gp16 protein, may interact with a host protein to facilitate the transport of the P22 DNA across the cytoplasmic membrane of the host. With this in mind, the 6xHis-S*tagged pilot proteins were used to "fish out" the host factor by performing two consecutive purifications and then analyzing the proteins that co-eluted with the pilot proteins. In other cases, a cross-linker was also added to strengthen or stabilize the protein-protein interaction between the pilot protein and the host factor. Finally, a technique

called Virus Overlay Protein Blot Assay (VOPBA) was employed to isolate host membrane proteins capable of specifically interacting with the pilot proteins.

Materials and Method

Plasmids and bacterial strains. All bacterial strains came from the lab collection unless stated otherwise. *Salmonella* Typhimurium strain, MST 1757 [*leuA414*(Am) Fels2$^-$] was used as a control for the transduction assay and for propagation of relevant P22 phage strains. The Tn*phoA* transposon was packaged from MST 2002 [*hisC10091*::Tn*phoA his*(*H* or *A*)*9556*::MudP22/pJS28 plasmid for P22 tail carries Amp^R; alias TT15089] and delivered into the recipient strain MST2003 [DEL(*his-cob*)*907* DEL(*phoN*)*65*; alias TT14543]. The r $^-$m$^+$ *Salmonella* Typhimurium strain, MST 997 [*hsdL6 hsdSA29 ilv-452 metA22 trpC2 metE551 xyl-404 fla-66 rpsL120* H1-b H2-e,n,x (Fels2$^-$) nml *galE550*; alias TR5878] was used to transfer plasmids from *E. coli* to *Salmonella*. MST 3848 [LT2 *pir*$^+$/pRL27e] was the source of the mini-Tn*5* transposon. The pRL27 plasmid, has a mini-Tn*5* with *aph* (Kanamycin resistance gene) in the transposon. It also contains an *ori* R6K origin of replication. The hyperactive transposase is provided constitutively under the *tetA* promoter (99).The T-POP strain MST 4208 [*proBA47*/F'128 (*pro*$^+$ *lac*$^+$) *zzf-3834*::Tn*10dTet* (DEL20 DEL25)] was the source of the T-POP template DNA for cloning the T-POP transposon. The T-POP transposon was cloned into the EZ-Tn*5* pMOD-2<MCS> transposon construction vector from Epicentre using *Sma*I and *Hind*III. The *Salmonella mutD* mutator strain MST 3271 [*leu414*(Am) Fels2$^-$ *supE dnaQ*::Tn*10*] was used to propagate and create mutations in the P22 *virB3* phage DNA. The pET30 Enterokinase/Ligation-Independent Cloning (Ek/LIC) vector, pET26 vector and

the DE3 (BL21) *E. coli* expression host cell were purchased from Novagen. The *Salmonella* expression host, MST 4190 *leuBCD485 trp*::[Spc^R T7 gene 1 (T7 RNA polymerase) *lacp lacI*] was used instead of the *E. coli* expression host unless stated otherwise. The DH5α *E. coli* strain EM 1252 was used to maintain plasmids with cloned genes of interest.

Phage strains. P22 HT105/1 *int-201*, (a high transducing derivative of phage P22 carrying a mutation in the *int* gene which prevents integration of the P22 genome), was used for all transductions in *Salmonella*. P22 *virB3* is a lytic phage that has mutations in the *Vx* (O_R operator) and *K5* (O_L operator) binding sites for the c2 repressor. P22-64 [*virB3 sieA44 m44*] is a lytic strain of P22 that has a mutation in *sieA* in addition to mutations in the DNA binding sites for the c2 repressor. The mutation in *sieA* prevents the exclusion of superinfecting DNA from occurring. This ensures that all host cells are lysed even in the unlikely event that P22 integration occurs. The mutations in the DNA binding sites for the c2 repressor also cause P22 to be lytic. The inability of the c2 repressor to bind operators on the phage DNA and establish lysogeny directs P22 to proceed to the lytic cycle. All P22 strains and phage KB1 were obtained from the lab phage collection and were propagated on *Salmonella* Typhimurium MST 1757 unless noted otherwise. Phage Ffm was the generous gift of Prof. Ken Sanderson of the University of Calgary and was propagated on MST 2817 [*hsdL6 hsdSA29 hsdSB121 galE856 xyl-404 leu-3121 metA22 metE551 trpC2 ilv-452 rpsL120*]. Phage Felix-O1 (FO) was the generous gift of Prof. McConnell of Point Loma Nazareth University and was propagated on MST 2627 [*rfc-458* ; alias SL428(SGSC# SGSC68) Rfc$^-$; P22^R Ffm^R FO^S].

Phage propagation. An overnight culture of the host cell was subcultured 1:100 in LBEDO broth. The LBEDO media was prepared according to Maloy (110). The bacterial hosts were grown to 100 Klett units by shaking at 37°C. An isolated plaque was added to the actively growing bacterial culture, and the phage were allowed to adsorb to the host cells in a 37°C water bath for 15 min without agitation. The culture was then reincubated for another 3 hr with rotation or shaking until complete lysis. Several drops of chloroform were added and the culture tube was vortexed to artificially lyse cells that have not been lysed by the phage but may harbor mature phage progenies. The chloroform was allowed to settle to the bottom of the culture tube for at least 10 min before the supernatant was transferred to a sterile phage vial with chloroform. Phage lysates were stored at 4°C.

Chemicals. N-Methy-N'-Nitro-N-Nitrosoguanidine (MNNG) was purchased from AccuStandard (New Haven, CT). Diethylsulfate (DES), Hydroxylamine (HA), Isopropyl-β-D-thiogalactoside (IPTG), 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), sarkosyl, lysozyme, chloroform, mitomycin C, and Triton X-100 were purchased from Fisher Scientific. Dithiothreitol (DTT) was obtained from Sigma. Rabbit anti-6xHis antibody was purchased from Santa Cruz Biotechnology. Goat anti-rabbit antibody was purchased from Jackson ImmunoResearch. His-bind affinity column purification kit, S*tag agarose and benzonase were purchased from Novagen. The BCA Protein Assay kit and the cross-linkers Dimethyl Suberimidate•2 HCl (DMS) and Dithiobis-succinimidylpropionate (DSP) were obtained from Pierce Biotechnology, Inc.

Culture media. Minimal E media and Evans Blue Uranine (EBU) agar plates were prepared as described in Maloy *et al.* (111).

Tn*phoA* *mutagenesis*. The procedure for Tn*phoA* packaging, delivery and mutagenesis was described by Maloy *et al.* (111). Tn*phoA* was packaged into P22 transducing particles by using the hybrid phage called Mud-P22 (Figure 4.2). An incomplete genome of P22 is sandwiched between the R and L insertion sequences (IS) of the Mu phage. This P22 derivative has a deletion of *int* and *xis* which prevents its integration and excision. Thus, when integrated into the chromosome, the Mud-P22 is "locked-in". Upon induction with mitomycin C, Mud-P22 undergoes multiple rounds of DNA replication without excision from the bacterial chromosome and DNA replication extends into adjacent chromosomal DNA in both directions. This results in an "onion-skin"–like amplification of the chromosomes surrounding the "locked-in" prophage. Once sufficient "pac" nuclease (encoded by gene 3) has accumulated, the enzyme will cut at the pac site and package adjacent chromosome in one direction into P22 transducing particles. The P22 transducing particles that have packaged Tn*phoA* were then used to transduce a *Salmonella* Typhimurium recipient strain that is deleted in *his* to prevent Tn*phoA* from recombining with the recipient strain (Figure 4.3). $P22^R$ mutants were selected from the pool of Kanamycin resistant transductants by replica plating on EBU plates supplemented with lytic P22 particles (e.g. P22-64). $P22^R$ mutants with mutations in the LPS biosynthetic genes were eliminated by testing for sensitivity to phage Ffm and phage Felix-O1.

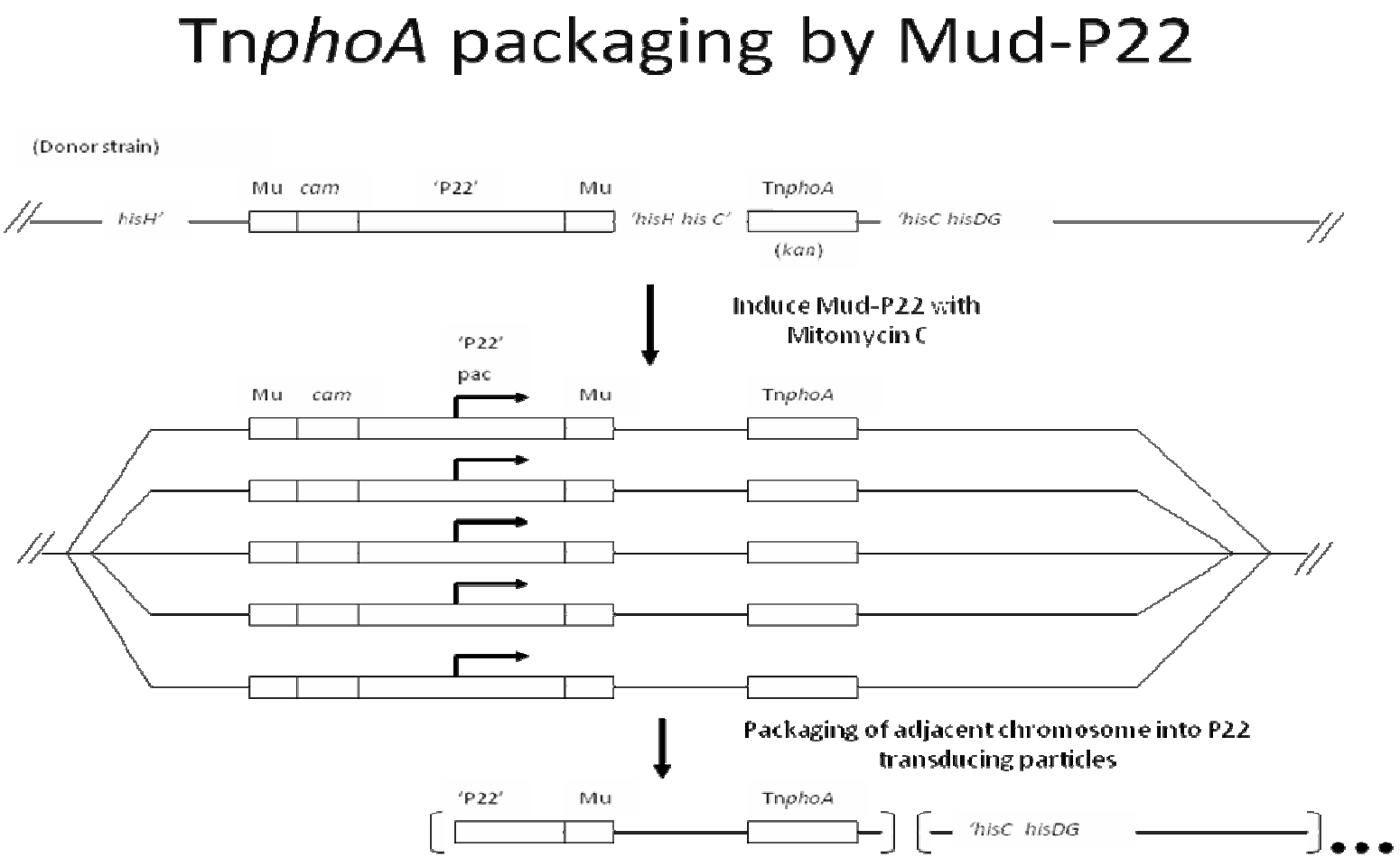


Figure 4.2: *The hybrid phage Mud-P22*. Mud-P22 was used to package the Tn*phoA* transposon which avoided the use of infective P22 particles in transposon delivery.

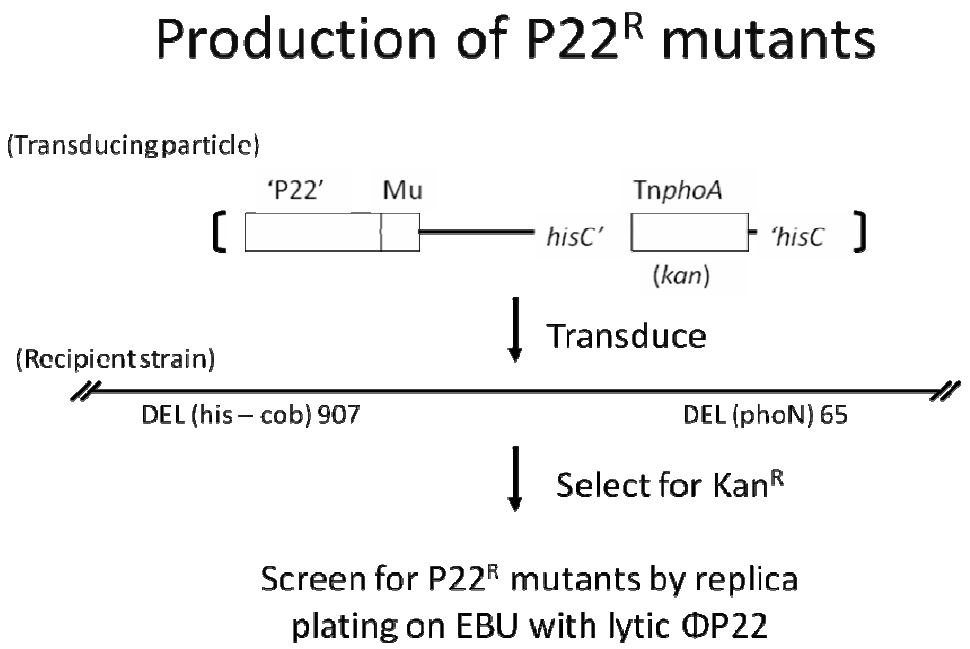


Figure 4.3: *Generation of P22^R TnphoA mutants*. Mud-P22 transducing particles carrying the Tn*phoA* transposon were used to transduce a *his*$^-$ *phoN*$^-$ recipient strain. Tn*phoA* mutants were selected on Kanamycin plates and replica plated on EBU plates supplemented with lytic P22 to select for P22^R mutants.

Tn5 mutagenesis. The mini-Tn*5* transposon was cloned into a plasmid (pRL27) that also has the transposase gene under the control of a *tetA* promoter. The *tetAp::tnp* gene construct is located outside of the transposon so that after transposition, the transposase gene is lost, resulting in stable transposon insertions. The transposon, designated Tn*5*-RL27 (99), is comprised of optimized Tn*5* inverted repeats flanking the *aph* gene from Tn903, encoding Kanamycin resistance, as a selectable marker and the plasmid R6K DNA replication origin (*ori* R6K) to facilitate subsequent cloning of transposon insertion mutants. Also, because *ori* R6K requires the pi (π) protein encoded by the *pir* gene, these plasmids are incapable of replication

in hosts that lack *pir*. Thus, after the plasmid has been introduced by electroporation into non-*pir* host cells, Kanamycin resistant transformants can be obtained only if the transposon inserts into the recipient genome. *Salmonella pir* ⁻ recipient cells were electroporated to introduce the Tn*5*-RL27 transposon and Tn*5*-containing electroporants were selected on Kanamycin plates. Tn*5* insertion mutants were replica plated onto EBU plates supplemented with lytic P22 particles to select for $P22^R$ mutants. P22 resistant mutants with truncated LPS were again eliminated as before.

T-POP mutagenesis. The procedure for T-POP transposon mutagenesis was based on Rappleye and Roth (135). The T-POP transposon was PCR-amplified from the *Salmonella* strain (MST 4208) carrying the F' plasmid with the T-POP insert and was cloned into the pMOD vector (Epicenter) using *Sma*I and *Hind*III. The T-POP transposon PCR product was cloned between the hyperactive 19bp Mosaic Ends (ME) which are specifically and uniquely recognized by the EZ-Tn*5* Transposase (58). Another round of PCR amplification was performed using primers that would amplify the T-POP transposon starting from the hyperactive Mosaic Ends flanking both ends of the T-POP transposon. Initially, the transposase was combined with the T-POP transposon to allow the enzyme to bind the mosaic ends of the T-POP transposon forming a complex called a "transposome". The transposome was then introduced into the host cell by electroporation which allowed the T-POP transposon to randomly hop into the chromosome of the host cell by a multi-step "cut and paste" transposition reaction catalyzed by the transposase (Figure 4.4). After electroporation, the mutagenized cells were plated on minimal media + Tet (4 µg/ml). The Tet^R transformants were replica plated on minimal media alone to assess whether the T-

POP transposon disrupted any essential gene downstream of the insertion site. The transformants were also replica plated on EBU plates supplemented with lytic P22-64 to select for $P22^R$ mutants. $P22^R$ mutants with mutations in the LPS biosynthetic genes were eliminated as before.

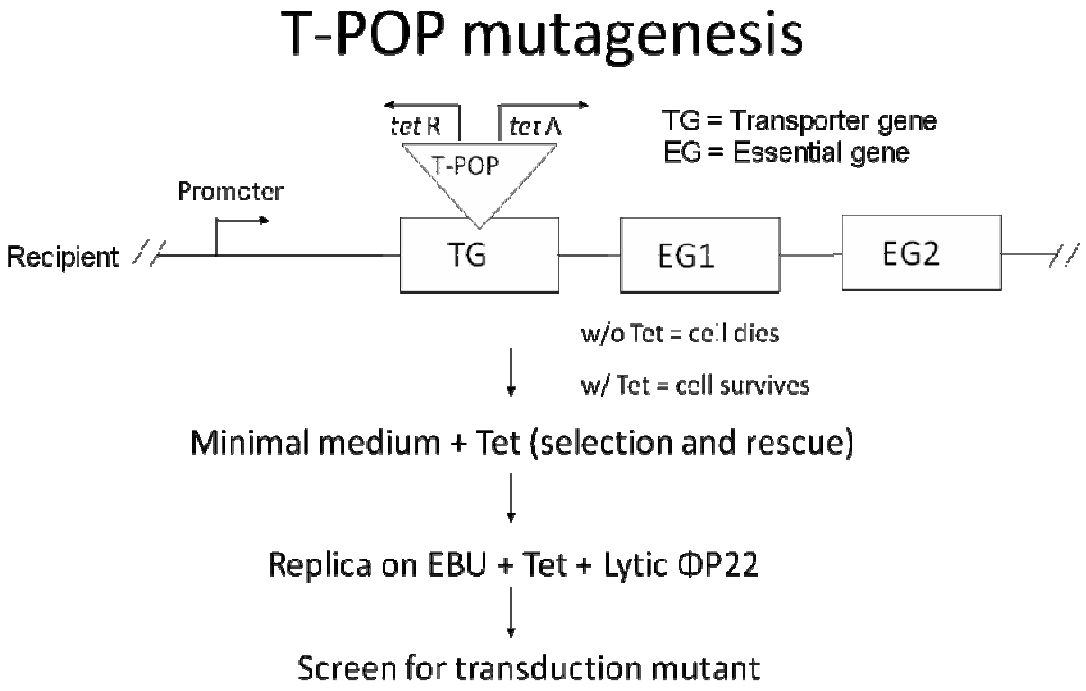


Figure 4.4: *Selection and rescue of T-POP $P22^R$ mutants*. T-POP insertions can generate Tet-conditional mutants. The T-POP transposon has the divergently transcribed *tetR* and *tetA* genes flanked by IS*10* inverted repeats. To generate Tet-conditional mutants, insertions of the T-POP element can separate essential genes from its promoter while inactivating the nonessential gene of interest (e.g. Transporter gene) upstream of the essential genes in the same transcription unit. Mutants are Tet-dependent because induced expression of the outward-pointing *tetRA* promoters in the presence of Tetracycline (w/ Tet) can allow the transcription of adjacent, essential genes. In the absence of Tetracycline (w/o Tet), transcription initiated from the TetR-repressed outward-pointing *tetRA* promoters is not sufficient to express adjacent, essential genes at levels required for growth.

Diethysulfate (DES) mutagenesis. Protocol for Diethysulfate (DES) mutagenesis was based on Roth (150). Briefly, 50 µl of DES was added to a 10 ml screw-capped test tube containing 2.5 ml of E medium with no carbon source. The tube was vortexed and incubated in a 37°C water bath for 10 min to form a saturation solution of DES. Then 50 µl of an overnight culture of a *Salmonella* strain to be mutagenized was added to the aqueous phase of the saturated DES solution. 50 µl of the overnight culture was also added to 2.5 ml of E medium without DES as a control. Tubes were incubated for 50 min in a 37°C water bath without shaking to allow mutagenesis to occur. 50 µl was removed from the mutagenized and control tubes and subcultured into 2 ml of LB broth for overnight recuperation at 37°C. The mutagenized and control cultures were diluted to 10^{-6} using 0.85% NaCl and 0.1 ml of the dilution was plated on an LB plate which was incubated at 37°C overnight. For direct genetic analysis, the mutagenized *Salmonella* cells plated on LB plates were replica plated on EBU plates supplemented with the lytic phage P22-64 to select for $P22^R$ mutants. For suppressor analysis, the mutagenized *Salmonella sieA*$^+$ lysogens were replica plated on EBU plates supplemented with the lytic phage P22 *virB3* to screen for P22-sensitive ($P22^S$) mutants. Mutagenized and non-mutagenized cells were plated on streptomycin plates to assess the level and frequency of DES mutagenesis. Mutagenized cells repeatedly had 10 to 100 times more streptomycin resistant colonies than the non-mutagenized cells.

Nitrosoguanidine (MNNG) mutagenesis. A 1 ml aliquot of a *Salmonella* Typhimurium culture grown in LB broth to 100 Klett units (10^9 cfu/ml, mid-logarithmic phase) was centrifuged at 10,000 x g for 1 min using a tabletop centrifuge and resuspended in 2 ml of TM buffer (0.05 M Tris; 0.05 M maleic acid; pH adjusted to 6

with NaOH). The resuspended bacteria were centrifuged again and resuspended in 2 ml of TM buffer which contained 100 µg/ml of MNNG (diluted from a freshly-made 5 mg/ml stock of crystals dissolved in TM buffer). The mutagenesis mixture was incubated for 15 min at 37°C with shaking to continue the active growth of the culture. The bacteria were then pelleted again and resuspended in 1 ml of LB broth. A 100 µl aliquot of mutagenized cells was taken and saved for determination of cell viability by serial dilution, using 100 µl of unmutagenized culture as a control. Another 400 µl aliquot of mutagenized cells was then removed, diluted 10-fold to 4 ml using LB broth, and 100 µl aliquots were plated onto each of 40 EBU agar plates that had been seeded with virulent P22-64. These plates were incubated overnight at 37°C to select $P22^R$ mutants.

Screening for $P22^R$ mutants with intact LPS. $P22^R$ mutants with mutations in the *rfa* and *rfb* LPS biosynthetic genes were screened out by testing their sensitivity to phage Ffm and phage Felix-O1 (Figure 4.5). A *Salmonella* Typhimurium cell with wild-type *rfa* and *rfb* genes is sensitive to P22 and Felix-O1 but resistant to phage Ffm. Truncation of the O-antigen residues due to mutations in the *rfa* or *rfb* LPS genes makes the bacterium sensitive to phage Ffm and resistant to Felix-O1. Hence, $P22^R$ mutants that are Ffm-resistant and Felix-O1-sensitive can be inferred to have an intact LPS *core*. $P22^R$ mutants with a specific mutation in the *rfc* LPS gene cannot be ruled out by phage Ffm and phage Felix-O1. *rfc* mutants are resistant to Ffm and sensitive to Felix-O1 because they have a single O-antigen residue. They were eliminated by performing an adsorption assay or by visualizing their LPS profile on an SDS polyacrylamide gel stained with a silver stain. The adsorption kinetics and the

LPS profile of the P22^{R} mutants were directly compared to the adsorption kinetics and LPS profile of *Salmonella* cells with intact LPS.

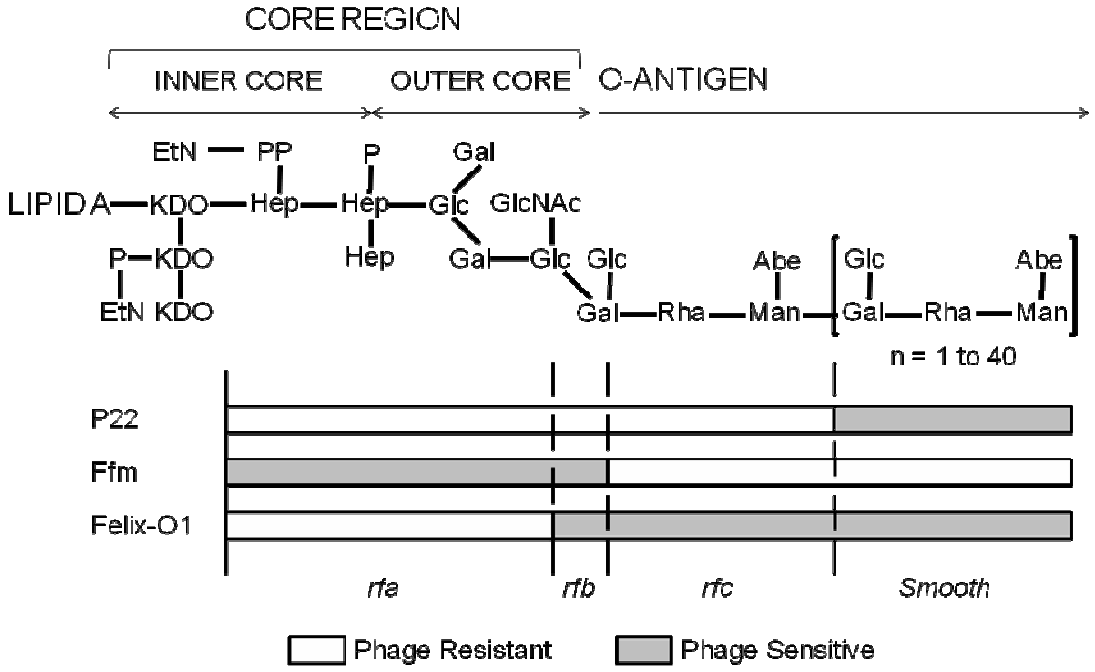


Figure 4.5: *Phage sensitivity of lipopolysaccharide mutants*. P22^{R} mutants were tested for their sensitivity to phage Ffm and phage Felix-O1. Mutants that were Ffm-sensitive and Felix-O1-resistant were eliminated because they were presumed to have a truncation in their LPS. Mutants that were Ffm-resistant and Felix-O1-sensitive were presumed to have at least one O-antigen residue in their LPS structure and were therefore further tested to determine if they had a wild-type copy of the *rfc* LPS gene. Figure modified from Lawes (1997).

One-step growth curve. The one-step growth curve was based on the protocol developed by Ellis and Delbrück (43). This experiment synchronizes an infected population of cells for one complete cycle of cell lysis. A one-step growth curve was constructed to classify the P22^{R} mutants based on where they are blocked in the phage infection process. To construct the one-step growth curve, wild-type *Salmonella* Typhimurium, an LPS mutant strain of *Salmonella* Typhimurium and the

$P22^R$ mutants were cultured in liquid medium to a concentration of 10^9 cfu/ml (Figure 4.6). The cultures were infected with lytic P22 at an MOI of 0.01, incubated at 37°C for 10 min to allow adsorption and then diluted 1:20 with pre-warmed LB broth. Infected cultures were incubated at 37°C for 1 hr. Aliquots were removed every 10 min and the titer of unadsorbed phage in the supernatant was determined for each time point by serially diluting the supernatant and applying 10 µl spots of the different dilutions onto an EBU plate topped with soft agar containing 100 µl of host cells known to be sensitive to the phage being titered.

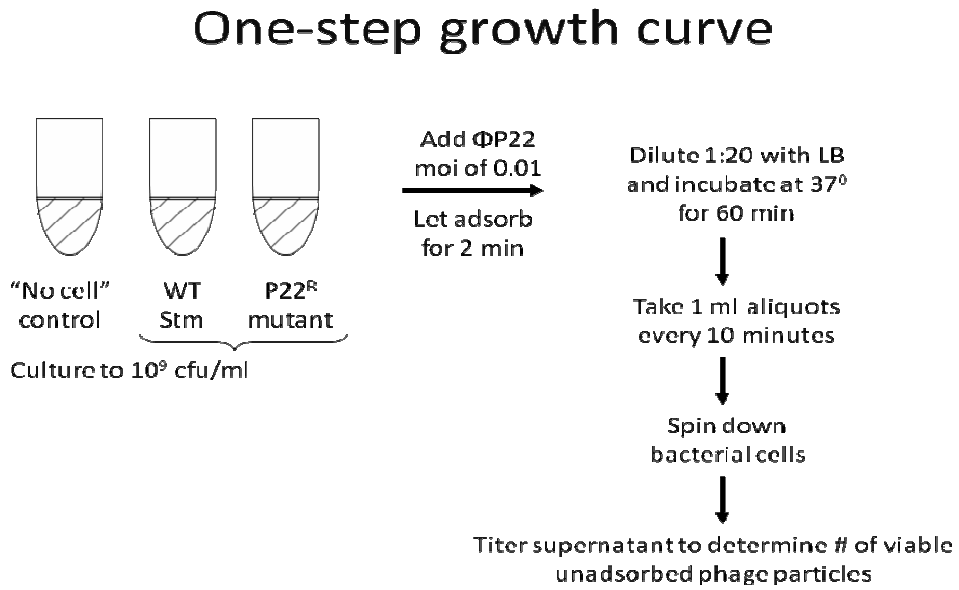


Figure 4.6: *The one-step growth curve*. The one-step growth curve is a useful tool in dissecting which stage of the P22 infection process the phage is prevented from infecting the host cell. This is achieved by counting the number of unadsorbed phage particles in different time points of the phage infection process until one entire round of burst or host cell lysis is completed.

Adsorption assay. The adsorption assay is an abbreviated version of the one-step growth curve. A 100 µl aliquot was taken before the phage was added to the

host cells and another aliquot was taken after the phage has adsorbed to the host cells for 10 min at 37°C. These two time points were sufficient to determine if the phage particles were able to adsorb to the host. The titer of unadsorbed phage in the supernatant was determined in the same manner as described in the procedure for the one-step growth curve.

The transduction assay. The P22 transduction protocol was based on the protocol of Maloy (110). Briefly, P22 HT105/1 *int-201* was grown on a strain of *Salmonella* Typhimurium which harbors a copy of the Tetracycline-resistance cassette in the bacterial chromosome (Figure 4.7). The P22 transducing particles which have successfully packaged the Tetracycline-resistance cassette were serially-diluted before using them as donors in transducing the $P22^R$ mutants or *Salmonella* Typhimurium MST 1757. 200 µl of an overnight culture of the recipient strain was incubated with 200 µl of the different dilutions of the P22 transducing particle in a 37°C water bath for 15 min to allow for adsorption before the mixtures were plated on Tetracycline (20 µg/ml) plates. Plates were incubated overnight in a 37°C incubator. The number of transductants was counted the next day to compare the transduction efficiency of the $P22^R$ mutants to the transduction efficiency of *Salmonella* Typhimurium MST 1757.

Mutagenesis by mutD strain. Mutations in genes involved in DNA repair often have a mutator phenotype; that is, the resulting defect in DNA repair increases the frequency of mutagenesis (111). For example, mutations in the *dnaQ* gene (also called *mutD*), which is required for the proofreading function of DNA polymerase III, and mutations in the *mutH*, *mutL*, and *mutS* genes, which eliminate methyl-directed mismatch repair, all confer powerful mutator phenotypes. The mutation frequency of

DNA passed through mutator strains is greatly enhanced. Since efficient mutator strains generally produce “sickly” colonies because of the accumulation of deleterious mutations in the dividing cells, there is a strong selection for suppressors of the mutator phenotype, which can be seen as healthy, fast growing colonies. Therefore, when mutator strains are used, healthy-looking colonies have to be avoided because they may have lost the mutator phenotype.

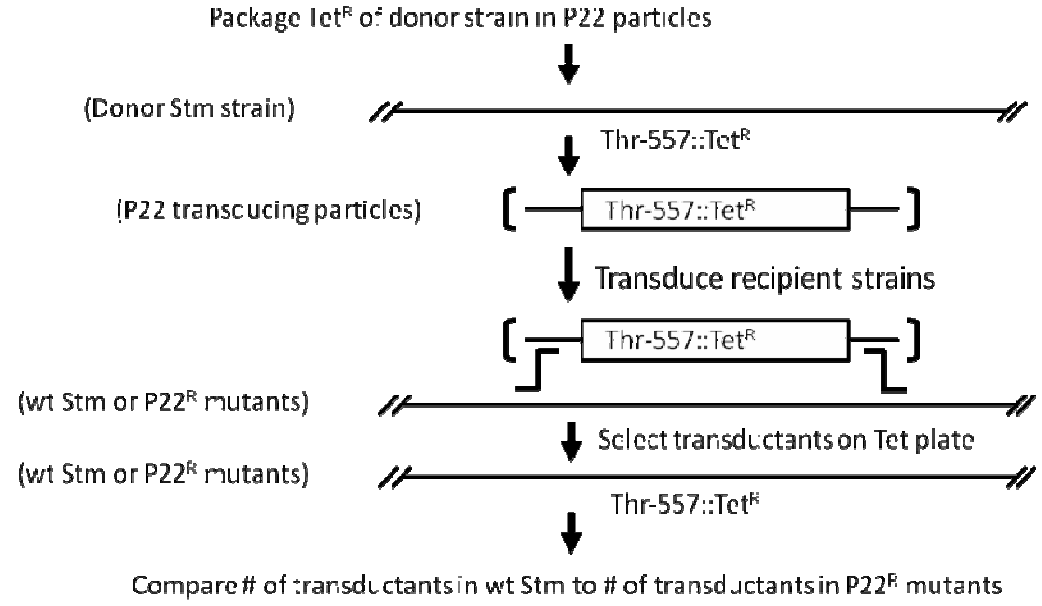


Figure 4.7: *The transduction assay*. The Tetracycline selectable marker was packaged in high transducing P22 HT105/1 *int-201* particles. The transducing particles were used to infect the adsorbing P22^R mutants to determine the degree in which the mutants would be able to uptake the Tet resistance gene carried by the phage. Transductants were plated on a Tet plate and the efficiency of transduction of the P22^R mutants was compared to the efficiency of transduction of *Salmonella* Typhimurium MST 1757.

P22 *virB3* was propagated on the *Salmonella* Typhimurium *mutD* strain for suppressor analysis. Suppressor analysis may provide some information whether there are phage-encoded factors that contribute to the transport of phage DNA across

the cytoplasmic membrane of the host cell. Serial dilutions of the mutated P22 *virB3* phage particles were then used to infect both MST 1757 and a P22 *sieA*$^+$ lysogen of *Salmonella* Typhimurium by adding 10 µl spots of the mutated phage lysate onto EBU plates with top agar containing the respective host cells. A P22 *sieA*$^+$ lysogen would normally be resistant to an incoming P22 *virB3* DNA because the SieA protein has the ability to prevent entry of the incoming DNA by either blocking the DNA per se or by inhibiting the function of any of the pilot proteins guiding the DNA across the cytoplasmic membrane. If the mutated P22 *virB3* DNA makes it across the cytoplasmic membrane of the P22 *sieA*$^+$ lysogen (e.g. plaques are formed), then that would mean that the mutation in the P22 *virB3* DNA allowed the DNA to bypass the exclusionary function of the SieA protein.

Hydroxylamine (NH_2OH) mutagenesis. P22 *virB3* was also mutagenized using hydroxylamine for suppressor analysis. When used *in vitro*, hydroxylamine reacts with cytosine, converting it to a modified base that pairs with adenine (110). This has two consequences: (1) hydroxylamine only produces G:C to A:T transitions, and (2) mutations induced by hydroxylamine cannot be reverted with hydroxylamine. In addition, hydroxylamine gives a high ratio of mutagenic to lethal events. The extent of mutagenesis can be monitored by following the mutagenesis of phage present in the lysate. The protocol outlined in Maloy (110) was followed to mutagenize P22 *virB3* using hydroxylamine. Wild-type P22 was also mutagenized at the same time P22 *virB3* was mutagenized to assess the efficiency of hydroxylamine mutagenesis. Mutagenized wild-type P22 produces more clear plaques than the non-mutagenized wild-type P22. The hydroxylamine-mutagenized P22 *virB3* particles were then used

for suppressor analysis in much the same way as the P22 *virB3* particles that were mutagenized using the *mutD* strain of *Salmonella* Typhimurium.

Plaque formation assay. Mutagenized and non-mutagenized P22 *virB3* particles were serially diluted and 10 µl of each dilution was spotted onto an EBU plate with top agar containing 100 µl of *Salmonella* Typhimurium MST 1757 or a P22 *sieA*$^{+}$ lysogen strain.

Vector Construction. DNA sequences of genes coding for the P22 pilot proteins (gp 7, 16 and 20) were obtained from genbank. The P22 DNA template was extracted from concentrated P22 lysate as previously described (111). Forward and reverse primer sequences used to amplify gene 7 were: Forward (5' to 3') GACGACGACAAGATGAAAGGCGGTAAAGGTGGCGCAGATAAAAGC; Reverse (5' to 3') GAGGAGAAGCCCGGTTTAAAACAACGAGCCAAGCAGACCAATACC. Forward and reverse primer sequences used to amplify gene 16 were: Forward GACGACGACAAGATGAAAGTTACCGCTAATGGCAAGACATTC; Reverse GAGGAGAAGCCCGGTCTACTGCCGGGTAGCTTCGTTAGCTAAAAG. Forward and reverse primer sequences used to amplify gene 20 were: Forward GACGACGACAAGATGGCTACGTGGCAGCAGGGCATTAATTCAGGT; Reverse GAGGAGAAGCCCGGTTTATTCCACCGTGAATTTAATGCCAGATTT.

The amplified gene sequences were cloned into the pET30 Ek/LIC expression vector from Novagen putting the 6xHis and the S*tag on the N-terminus of the expressed protein of interest. The gp16 gene was also cloned into the pET26 vector using *BamH*I and *Xho*I. The pET26 vector carries an N-terminal *pelB* signal sequence for potential periplasmic localization and a C-terminal 6xHis-tag sequence. The

presence of the appropriate inserts in the pET vectors was confirmed by PCR, restriction digests and DNA sequencing. Expression vectors with the correct inserts were electroporated into either a DE3 *E. coli* expression host or a *Salmonella* Typhimurium expression host cell line (MST 4190) and plated on LB agar plates with the appropriate selection marker. Antibiotic concentration used is as follows: Kanamycin 50 µg/ml, Ampicillin 100 µg/ml.

Protein Expression and Purification. BL21 cells harboring the specific expression vector with the appropriate insert were inoculated into 100 ml of LB broth with the appropriate antibiotic concentration and incubated in a 37^{o}C shaker until mid-exponential growth. IPTG was added to a final concentration of 1 mM to induce protein expression and the cultures reincubated for an additional 3 hr in a 30^{o}C shaker. The cells were then centrifuged at 3842 x g for 30 min at 4^{o}C. The supernatant was removed and the pellet resuspended in 4 ml of lysis buffer (50 mM NaH_2PO_4, pH 8, 300 mM NaCl, 10 mM Imidazole, 10 mM CHAPS). Lysozyme was added to 0.5 mg/ml and 100 units of benzonase was also added to reduce viscosity of the lysate. The lysate was incubated in ice for 30 min with occasional swirling and then subjected 3 times to sonication using the Fisher Scientific ultrasonic dismembrator Model 100. The sonicated lysate was centrifuged at 11000 x g for 30 min at 4^{o}C. The supernatant was added to a column containing 200 µl bed volume of nickel-charged His-bind resin (Novagen) and samples eluted at a flowrate of ca. 1 ml/min. The resin was washed three times with 1X binding buffer (50 mM NaH_2PO_4, pH 8, 300 mM NaCl, 20 mM Imidazole, 10 mM CHAPS) and washed twice with 1X wash buffer (50 mM NaH_2PO_4, pH 8, 300 mM NaCl, 60 mM Imidazole, 10 mM CHAPS) before the 6xHis-tagged ejection protein was eluted three times with 200 µl

each of 1X elution buffer (50 mM NaH_2PO_4, pH 8, 300 mM NaCl, 1 M Imidazole, 10 mM CHAPS). The 1 M imidazole was removed from the eluted ejection protein by using a centricon filter with a molecular weight cut off of 5 kDa and by washing the ejection protein with 20 mM Tris buffer, pH 8, 100 mM NaCl, 0.1% Triton X-100. Secondary purification was performed on an affinity column loaded with the S*tag agarose. Eluted proteins were filtered again in a centricon filter to remove all traces of $MgCl_2$ present in the elution buffer. Protein concentration was determined using the BCA Protein Assay kit from Pierce. The purified proteins were used in co-eluting host membrane proteins, cross-linking host factors or in virus overlay protein blot assay.

Western Blot. PVDF membranes were probed with rabbit anti-6xHis antibody at a 1:1500 dilution. The goat anti-rabbit secondary antibody conjugated with horseradish peroxidase was used at a 1:10,000 dilution. Bands were visualized using chemiluminescence detection (Supersignal; Pierce) and exposed to X-ray film (Kodak).

Membrane protein purification. Crude membrane extract of *Salmonella* Typhimurium was prepared according to Muro-Pastor (121). When purified membrane proteins were needed, the membrane extract was solubilized in 1% Triton X-100 and passed through a 23 gauge syringe needle dropwise at least 5 times before ultracentrifuging the membrane vesicles at 227640 x g for 3 hr using a Beckman Vti 65.2 Class H rotor. Most of the membrane proteins remained in the supernatant surrounded by the micelles formed by the detergent. 1x PBS was substituted for 20 mM Tris in the preparation of the membrane extract when the membrane extract was to be used in cross-linking with the gp16 protein. Tris and other primary amines react with the cross-linkers and compete with the proteins being

cross-linked. The membrane extract was solubilized in 2% sarkosyl to specifically obtain cytoplasmic membrane proteins.

Cross-linking 6xHis-gp16 to potential host factors. Protocol was adapted from Kawaguchi *et al.* (85). Specific details on the application of each cross-linker were followed per manufacturer's instructions. Briefly, membrane extract from *Salmonella* Typhimurium was added to 1 mg/ml of twice-purified 6xHis-gp16 protein and the mixture was incubated at room temperature with gentle shaking for 60 min to allow the gp16 protein to bind to host membrane proteins. The cross-linker was added at 30-fold molar excess to the mixture and the tube was incubated at room temperature for another 60 min. The cross-linking reaction was terminated by adding Tris at 50 mM final concentration. The mixture was solubilized in 1% Triton X-100 overnight at 4^{o}C with gentle shaking and the next day, the 6xHis-gp16 protein covalently linked to host membrane proteins was purified using a nickel column. Eluted materials were mixed with SDS-loading buffer and loaded on an SDS-PAGE without boiling for Western blot analysis. A variation of the cross-linking protocol was to cross-link the gp16 protein to purified total membrane proteins or to purified cytoplasmic membrane proteins which required solubilization of the membrane extract with 1% Triton X-100 or 2% sarkosyl, respectively, prior to the cross-linking step.

Virus Overlay Protein Blot Assay (VOPBA). The procedure for this assay was based on Koudelka *et al.* (93). Purified membrane proteins were ran on a 10% SDS polyacrylamide gel and transferred to a PVDF membrane. The membrane was incubated for two rounds of 10 min each in denaturing buffer consisting of 6 M guanidine-HCl, 2 mM EDTA, 50 mM DTT, and 50 mM Tris-HCl at pH 8.3. The PVDF

membrane was then incubated for 10 each at 4°C in renaturing buffer consisting of 10 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, 2 mM DTT, and 0.1% Triton X-100 at pH 7.3 with sequentially reduced amounts of guanidine-HCl, at 4 M, 3 M, 2 M, 1 M, and 0 M. Following the guanidine-HCl treatment, the membrane was blocked using 5% non-fat dry milk in renaturing buffer overnight at room temperature; this temperature was maintained for the rest of the procedure. The membrane was then cut into strips and each strip was incubated with a specific purified pilot protein suspension consisting of 1% non-fat dry milk, 5% glycerol, and 10 µg/ml of purified pilot protein for 90 min, followed by four 5 min washes in wash buffer consisting of 1x PBS with 0.2% Triton X-100. The membrane strips were probed with rabbit anti-6xHis for 60 min and then washed four times for 5 min each in wash buffer. The membrane strips were then probed with goat anti-rabbit for another 60 min followed by four 5 min washes in wash buffer. Bands were visualized using chemiluminescence detection (Supersignal; Pierce) and exposed to X-ray film (Kodak).

Results

The mechanism of translocation of an anionic macromolecule like the phage DNA of P22 across the barrier formed by a hydrophobic lipid bilayer such as the cytoplasmic membrane of *Salmonella* Typhimurium is not clearly understood. It is possible that the P22 DNA is using an available and easily accessible pre-existing host factor (e.g. transporter protein) to transport itself across the cytoplasmic membrane of the host cell. To isolate host factor(s) contributing to the translocation of phage P22 DNA into the host cytoplasm, genetic and biochemical approaches were employed.

I. Genetic Approaches

The genetic approach was conveniently attempted since the DNA sequences of *Salmonella* Typhimurium and P22 are known and a great deal of information is available about the host and the phage. In addition, genetic manipulation of either the host or the phage is relatively facile.

A. Direct Genetic Analysis

The direct genetic analysis was performed by randomly mutagenizing *Salmonella* Typhimurium using either a transposon or a chemical mutagen. $P22^R$ *Salmonella* Typhimurium mutants were selected by plating the mutagenized cells onto an EBU plate supplemented with the lytic P22-64 phage.

Resistance to P22 is caused by any or a combination of 4 possible outcomes of mutagenesis. The first outcome of mutagenesis that would cause P22 resistance is a mutation in genes involved in lipopolysaccharide (LPS) biosynthesis. There are over 30 genes involved in the synthesis of the LPS which makes this group of genes an easy target for either a transposon or a chemical mutagen. The second outcome of mutagenesis that would cause P22 resistance is a mutation in the gene that codes for the secondary receptor on the outer membrane of the host cell that is involved in the irreversible adsorption of the phage particle. Another outcome of mutagenesis that would render a host cell resistant to P22 infection would be a mutation in genes involved in the transport of the phage DNA across the cytoplasmic membrane. The last outcome of mutagenesis that would enable a host cell to resist P22 infection would be a mutation in genes involved in phage maturation.

$P22^R$ mutants appear as white colonies on EBU plates with lytic P22 particles while $P22^S$ mutants show up as blue colonies. $P22^R$ mutants were always cleaned on an EBU plate to screen out potential pseudolysogens. Pseudolysogens are cells that contain a copy of the phage genome in the form of an episome (a circularized phage genome not integrated in the bacterial chromosome) which can be diluted out during cell replication since the phage genome can only be inherited by one daughter cell. True nonlysogens would form white colonies while some pseudolysogens would form blue colonies on the EBU plates. Isolated white colonies were cross-streaked against P22-64 to confirm $P22^R$ phenotype.

$P22^R$ mutants that have a truncated or defective lipopolysaccharide (LPS) were initially eliminated by using phage Ffm and phage Felix-O1. The adsorption assay or LPS profiling was performed to eliminate any $P22^R$ mutant that had a mutation in the *rfc* LPS gene which would not be eliminated by either phage Ffm or phage Felix-O1. The remaining $P22^R$ mutants with intact LPS were classified based on which stage of the infection process the phage is blocked in its attempt to infect the host.

1. Transposon Mutagenesis

One of the transposons used in the isolation of the host factor was Tn*phoA*. The Tn*phoA* transposon was packaged by a hydrid phage called Mud-P22 to avoid the introduction of infective P22 particles during the delivery of the transposon into the recipient cells. Using infective P22 particles is not the best way to deliver a transposon into the recipient cell if the goal is to isolate $P22^R$ mutants. This is because the use of infective P22 particles can result in the creation of P22 lysogens

which are resistant to P22 infection due to their ability to express the superinfection exclusion protein (SieA). The SieA protein which localizes to the cytoplasmic membrane (66) is poised to exclude any superinfecting P22 DNA (173).

The transducing particles which contain the Tn*phoA* transducing fragment were used to transduce a recipient strain, allowing the Tn*phoA* transposon to hop anywhere into the bacterial chromosome of the recipient cell. The Tn*phoA* transposon contains a Kanamycin resistance cassette which facilitated the selection of the Tn*phoA* insertion mutants. A recipient strain that had a deletion mutation in the *his* gene was used to prevent recombination. The recipient strain also had a deletion in *phoN* to make certain that the only enzyme that could cleave 5-Bromo-4-chloro-3-indolyl-phosphate (XP) would be the phosphatase encoded by *phoA* of the Tn*phoA* transposon. Replica plating of the generated Tn*phoA* mutants on an XP plate allowed for a quantitative determination of the efficiency of Tn*phoA* transposition into the bacterial chromosome of the recipient cell. The efficiency of Tn*phoA* transposition was repeatedly calculated to occur at 30% efficiency. The Tn*phoA* mutants were also replica plated onto EBU plates supplemented with lytic P22-64 particles to select for $P22^R$ mutants.

Following selection of $P22^R$ mutants, the mutants were screened to eliminate mutants that had a truncation in their LPS. This process of elimination was initially performed by testing the sensitivity of the $P22^R$ mutants to phage Ffm and phage Felix-O1. Although testing for phage Ffm sensitivity should be theoretically adequate in eliminating *rfa* and *rfb* LPS mutants, it was necessary to test for phage Felix-O1 sensitivity to eliminate $P22^R$ mutants with a mucoid phenotype. The mucoid

phenotype causes P22 resistance due to a polysaccharide capsule that surrounds the bacterial cell (100).

All TnphoA transposon mutants had a mutation in LPS biosynthetic genes. After screening the $P22^R$ Tn*PhoA* mutants to look for mutants with intact LPS, it was deduced that all isolated $P22^R$ mutants had a mutation in their LPS biosynthetic gene (Figure 4.8A). LPS biosynthetic genes comprise a huge fraction of the entire *Salmonella* coding sequence which makes them easy targets for transposon insertion and re-insertion. Transposon mutants with an insertion in an essential gene would have been missed because of the lethality of the Tn*phoA* insertion. To avoid the issue of obtaining unstable transposon insertions, another transposon mutagenesis was performed using an alternative transposon.

All Tn5 transposon mutants had a mutation in LPS biosynthetic genes. The next transposon employed in the isolation of the host factor was a mini-Tn*5* transposon cloned into a plasmid that also contained the transposase (*tnp*) gene under the control of a *tetA* promoter. *pir*$^-$ *Salmonella* recipient cells were electroporated to introduce the Tn*5*-RL27 transposon and Tn*5*-containing electroporants were selected on Kanamycin plates. Tn*5* insertion mutants were replica plated onto EBU plates supplemented with lytic P22 particles to select for $P22^R$ mutants. $P22^R$ mutants with truncated LPS were again eliminated using phage Ffm and phage Felix-O1. The Tn*5* mutagenesis generated 181 $P22^R$ mutants (Figure 4.8B). Only 12 of these mutants were resistant to phage Ffm and not one was found to be sensitive to phage Felix-O1. Like the Tn*phoA* mutagenesis, the Tn*5*

Figure 4.8: *Transposon mutagenesis*. (A) All $P22^R$ mutants generated by Tn*PhoA* mutagenesis had a truncated lipopolysaccharide which made them resistant to P22 infection. (B) All $P22^R$ mutants generated by Tn*5* mutagenesis also had a truncated lipopolysaccharide. (C) All $P22^R$ mutants generated by T-POP insertion mutagenesis had a defect in their LPS biosynthetic genes.

A) Tn*phoA* mutagenesis

~4500 Kan^R colonies

↓

487 P22 resistant colonies

↓ Eliminated rfa and rfb mutants

32 Ffm resistant colonies

↓ Eliminated mucoid colonies

0 FO sensitive colonies

↓ Eliminated rfc mutants

All P22 resistant colonies = LPS mutants

B) Tn*5* mutagenesis

6150 Kan^R colonies

↓

181 P22 resistant colonies

↓ Eliminated rfa and rfb mutants

12 Ffm resistant colonies

↓ Eliminated mucoid colonies

0 FO sensitive colonies

↓ Eliminated rfc mutants

All P22 resistant colonies = LPS mutants

C) T-POP mutagenesis

~6000 Tet^R colonies

↓

350 P22 resistant colonies

↓ Eliminated rfa and rfb mutants

74 Ffm resistant colonies

↓ Eliminated mucoid colonies

11 FO sensitive colonies

↓ Eliminated rfc mutants

All P22 resistant colonies = LPS mutants

mutagenesis only resulted in P22^R mutants that had a mutation in genes involved in LPS biosynthesis.

Since all P22^R mutants generated by Tn*phoA* and Tn*5* transposon mutagenesis had a mutation in the LPS biosynthetic genes, it was speculated that the gene required for the uptake of P22 DNA is an essential gene and that the transposons used thus far interrupted the expression of essential genes downstream of the transposon insertion site. Transposons like Tn*phoA* and Tn*5* have a strong polar effect on the expression of genes downstream of the insertion site. This means that if there were essential genes downstream from the Tn*phoA* or Tn*5* transposon insertion site, those essential genes would not be expressed. This may have caused the actual number of P22^R mutants to be underestimated since recipient cells that had a transposon insertion would have died prior to the selection of P22^R mutants. To compensate for the strong polar effect produced by Tn*phoA* and Tn*5* and to rescue insertion mutants that have essential genes downstream of the transposon insertion site, the Tn*10*d(TPOP) transposon was used.

All T-POP transposon mutants had a mutation in LPS biosynthesis genes. After performing T-POP mutagenesis on *Salmonella* Typhimurium MST 1757 and screening for P22^R mutants with full-length LPS, it was found that all P22^R mutants had a defect in their LPS structure which rendered them resistant to P22 infection (Figure 4.8C).

The outcome of the T-POP mutagenesis would seem to suggest that either the host factor is indeed encoded by an essential gene or the uptake of phage DNA is dependent upon phage-encoded factors. The host factor could very well be encoded

by an essential gene since a T-POP transposon insertion in this gene would not result in cell survival even in the presence of Tetracycline. Assuming that all essential genes downstream of the T-POP insertion site were expressed in the presence of Tetracycline, then the only possibility that would not allow expression of an essential gene is if the insertion happens to be in the essential gene itself. An insertion in an essential gene itself cannot be tolerated by the host cell and will therefore not generate a Tet-conditional mutant.

2. Chemical Mutagenesis

Transposon mutagenesis entails inserting a huge fragment of DNA in the host chromosome. The transposons used in the transposon mutagenesis ranged in size from 2 to 5 Kb and the size of the DNA insertion may have prevented the isolation of the mutant of interest. Chemical mutagenesis, on the other hand, produces missense and conditional mutants rather than strictly null mutants as commonly occur with transposon mutagenesis. Chemical mutagenesis occurs not just randomly but in multiple regions of the bacterial chromosome. This could be useful in preliminary studies to assess whether or not a particular type of mutation can be isolated. This could also serve as a disadvantage since it would be difficult to attribute the mutant phenotype to a single mutation if multiple genes were affected by the mutagen. In spite of the disadvantages that come with using chemical mutagens, it is still a tool worth exploring to further investigate the possibility that a host factor exists.

The chemical mutagens used in the attempt to isolate host factors were Diethysulfate (DES) and Nitrosoguanidine (MNNG).

All DES mutants had a mutation in LPS biosynthetic genes. After performing the DES mutagenesis on *Salmonella* Typhimurium MST 1757 cells and screening for $P22^R$ mutants with untruncated LPS, all $P22^R$ mutants were found to have a defect in their LPS biosynthetic genes (Figure 4.9A).

The other chemical mutagen used in the isolation of the host factor was nitrosoguanidine (MNNG). MNNG is also an alkylating agent that acts on DNA at the growing DNA replication fork and often produces multiple mutations (111). It is a very powerful mutagen that is often useful for initial studies to determine whether or not it is possible to isolate a particular type of mutation.

MNNG-mutagenized *Salmonella* were replica plated on EBU plates supplemented with lytic P22-64 to select for $P22^R$ mutants. The confirmed $P22^R$ colonies were cross-streaked against phage Ffm and phage Felix-O1 to eliminate $P22^R$ colonies that had a truncation in their LPS. The LPS profile of each remaining $P22^R$ mutant was compared to the LPS profile of *Salmonella* Typhimurium MST 1757 on an SDS polyacrylamide gel (PAGE) to finally screen out any $P22^R$ mutant with a mutation in the *rfc* LPS biosynthetic gene.

Five nitrosoguanidine mutants with intact LPS were resistant to P22 infection. Although most of the $P22^R$ mutants created by nitrosoguanidine had a mutation in genes involved in LPS biosynthesis, 5 mutants showed a normal LPS profile when examined by SDS PAGE (Figure 4.9B and Figure 4.9C).

Figure 4.9: *Chemical mutagenesis*. (A) DES mutagenesis only produced P22^{R} mutants with defective LPS. (B) Random mutagenesis by nitrosoguanidine generated mostly P22^{R} mutants that had a mutation in the LPS biosynthetic genes which were eliminated using phage Ffm and phage Felix-O1. An LPS profile of the remaining 300 P22^{R} mutants revealed that 5 had untruncated LPS structure. (C) LPS profile of full-length LPS vs truncated LPS. The O-antigen length of the LPS structure of the different P22^{R} mutants was directly compared to the O-antigen length of the LPS structure of *Salmonella* Typhimurium MST 1757 by running the LPS samples on a Sodium Dodecylsulfate Polyacrylamide gel. The O-antigen residues were stained with silver following the protocol of Hitchcock and Brown (1983). The LPS structure of P22^{R} mutant # 27 (MST 5193) and mutant # 28 (MST 5194) showed a similar O-antigen striated pattern as the LPS obtained from *Salmonella* Typhimurium MST 1757. Strains of *Salmonella* Typhimurium that have a mutation in any of the LPS biosynthetic genes (rfa, rfb or rfc) do not show the O-antigen residues found on the surface of a wild-type *Salmonella* cell. The *rfc* mutant of *Salmonella* contains only one O-antigen residue which is not sufficient to allow phage adsorption. (D) One-step growth curve of the P22^{R} mutants. Although the P22^{R} mutants, mutant #9, 27 and 28 (MST 5192, MST 5193, and MST 5194, respectively) had normal adsorption kinetics, they were not lysed by the lytic P22 phage.

A)

DES mutagenesis

1400 DES mutants

↓

132 P22 resistant colonies

↓

0 Ffm resistant or FO sensitive colony

↓

All P22-resistant colonies = LPS mutants

B)

Nitrosoguanidine Mutagenesis

3600 P22 resistant colonies

↓ Eliminated rfa and rfb mutants

800 Ffm resistant colonies

↓ Eliminated mucoid colonies

300 FO sensitive colonies

↓ Eliminated rfc mutants

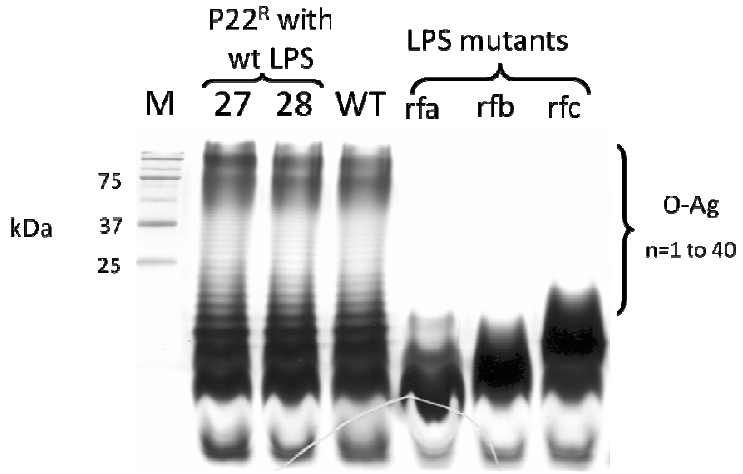
LPS profiling by SDS PAGE

↓

5 P22 resistant mutants with wt LPS

C)

LPS profile by SDS PAGE



D)

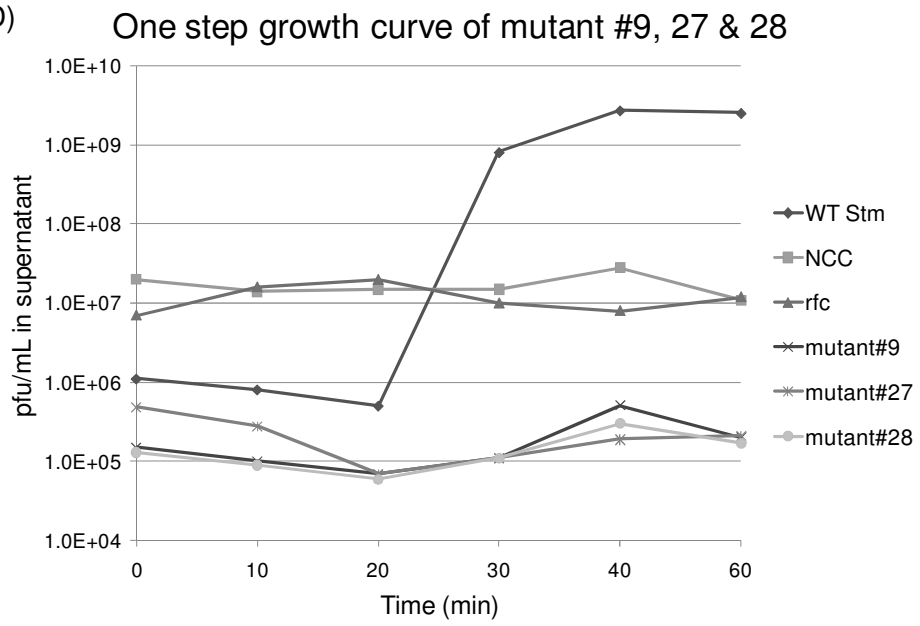


Figure 4.9: *Chemical mutagenesis*, Continued.

A one-step growth curve was performed on the P22^{R} mutants that showed intact LPS structure to confirm that they can undergo normal phage adsorption (Figure 4.9D). The one-step growth curve not only ruled out a mutation in adsorption but also indicated that the P22^{R} mutants had a mutation in transduction or phage maturation or both. The one-step growth curve of the P22^{R} mutants revealed that although they had normal adsorption kinetics, they could not be lysed by the lytic phage. Lysis of the mutant cells did not occur even after 60 min of incubation with the lytic P22 phage. Compared to the P22^{R} mutants, the *Salmonella* Typhimurium MST 1757 host cells showed release of the phage progenies starting at 20 min post-infection and continued bursting through 40 min post-infection. The *rfc* strain of *Salmonella* Typhimurium was expected to not adsorb P22 or produce any phage progeny since it lacks the required O-antigen residues on its LPS to bind the phage. The “No Cell Control” (NCC) sample was also expected to give a constant number of

unadsorbed phage particles from all collected time points since the original quantity of phage added had no means of replicating themselves without a host. It was possible that the original quantity of phage added lost some of its infectivity while being exposed to the warm temperature of 37^{o}C for 60 min but this possibility was ruled out by the fact that the titer of the original quantity of phage added remained constant in the “No Cell Control” sample.

Lysis of the P22^{R} mutants did not occur for a myriad of reasons. It is possible that the phage DNA was not able to enter the cytoplasm of the host where phage DNA replication occurs. It is also possible that the phage DNA gained access to the host cytoplasm but was unable to complete phage maturation due to mutations in genes involved in this particular stage of the phage infection process. A transduction assay was performed to assess if any of the P22^{R} mutants was incapable of transport of the phage DNA across the cytoplasmic membrane.

One nitrosoguanidine P22^{R} mutant had a defect in transduction. The P22^{R} mutants that were not defective in adsorption were subjected to a transduction assay to identify any mutants that had a defect or deficiency in transduction. The efficiency of transduction of these mutants was compared to the efficiency of transduction of *Salmonella* Typhimurium MST 1757. A decrease in transduction efficiency in any of the P22^{R} mutants would suggest that the P22^{R} mutant may have a mutation in a gene involved in the transport of the phage DNA across the cytoplasmic membrane of the host cell.

Subjecting the P22^{R} mutants to the transduction assay revealed one mutant with a deficiency in transduction (Table 4.1). Mutant #27 (MST 5193) was

Table 4.1: Identification of P22^R *Salmonella* Typhimurium mutants with normal adsorption but has a deficiency in transduction

Recipient Strains[(a)]	# transductants in 10^2 transducing particles[(b)]	# transductants in 10^3 transducing particles
MST 1757	745	99
Mutant #27 (MST 5193)	231 (31%)	30 (30%)

(a) Transduction efficiency of mutant #27 (MST 5193) was compared to the transduction efficiency of *Salmonella* Typhimurium MST 1757

(b) 10^2 and 10^3 dilutions of the transducing particles carrying the Tet^R cassette were used to transduce the recipient strains. Number of Tet^R transductants are shown. Number in parenthesis denotes transduction efficiency of mutant strain compared to transduction efficiency of *Salmonella* Typhimurium MST 1757 which was arbitrarily assigned 100% transduction efficiency.

found to be 70% deficient in the uptake of a Tet^R plasmid from the P22 transducing particle. Although the uptake of DNA is diminished in mutant #27 (MST 5193), the defect is only partial. One explanation as to why the transduction deficiency of mutant #27 may be incomplete is that there may be more than one gene involved in the transport of the phage P22 DNA across the cytoplasmic membrane, and only one of these genes was affected by the chemical mutagen. An alternative explanation is that the mutation induced by nitrosoguanidine caused a minor change in the conformation of the protein, retaining partial ability to translocate the P22 DNA. The incomplete inhibition of transduction could also be explained by the possibility that the transport of phage DNA does not have a strict requirement for the host factor. For example, if the specific host factor is available to bind the incoming phage DNA and if it is readily accessible then the phage DNA could use it, but if the host factor is not available, then the phage DNA would rely entirely on its pilot proteins to multimerize and form

the channel that can transport the phage DNA across the cytoplasmic membrane of the host cell.

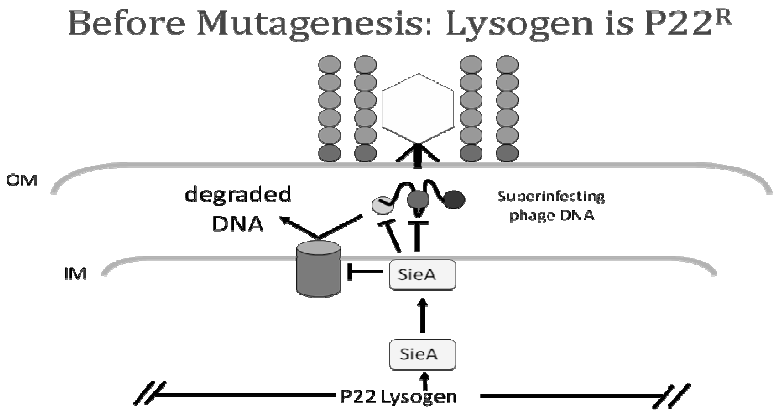
B. Suppressor Analysis

Suppressor analysis was the other genetic approach used to identify genes that may be involved in the transport of phage DNA across the cytoplasmic membrane of the host. Suppressor analysis employed a P22 lysogen of *Salmonella* Typhimurium. A P22 lysogen is established when the P22 genome integrates into the bacterial chromosome of the host by site-specific recombination that is mediated by the phage-encoded integrase protein together with other host proteins. Two of the many proteins expressed by the P22 prophage are the c2 repressor and the SieA proteins. The c2 repressor protein maintains the lysogeny state of the cell and the SieA protein which localizes to the cytoplasmic membrane excludes superinfecting P22 DNA from entering the P22 lysogen (66, 173). As long as the P22 SieA protein is not overwhelmed by too many P22 superinfecting DNA trying to cross the cytoplasmic membrane barrier of the P22 lysogen (i.e. MOI less than or equal to 10), the P22 lysogen remains resistant to P22 infection.

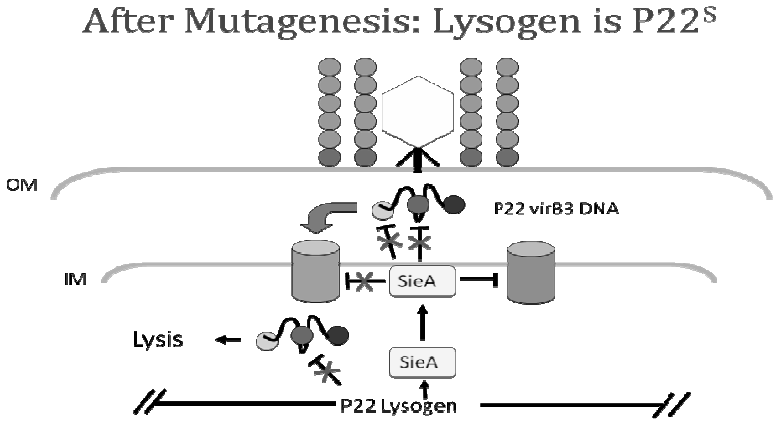
The mechanism of how the SieA protein exerts its ability to exclude superinfecting DNA is not known. The SieA protein could exclude incoming DNA by either binding to any of the pilot proteins guiding the DNA across the cytoplasmic membrane or by directly binding and blocking DNA uptake (Figure 4.10A). The SieA protein could also be associating and modifying a host protein that could potentially transport the incoming phage DNA across the cytoplasmic membrane. A mutation in the gene that encodes this host protein may render the P22 lysogen sensitive to P22

Figure 4.10: *Suppressor analysis*. (A) The SieA protein expressed by a P22 lysogen makes the cell resistant to P22 infection. One way SieA can exclude superinfecting P22 DNA is by modifying a host protein that can potentially transport the DNA across the cytoplasmic membrane of the host. A mutation in the gene that encodes this host protein may render the P22 lysogen sensitive to P22 infection. (B) Different ways of converting a P22 *sieA*$^{+}$ lysogen from being resistant to being sensitive to P22 *virB3* infection. A mutation in the channel protein (either a pre-existing host transporter or a phage-encoded channel protein) used by the SieA protein during superinfection exclusion could prevent the SieA protein from binding and repressing the channel protein. A mutation in any of the pilot proteins could also prevent the SieA protein from excluding the incoming phage DNA. Lastly, a mutation in the DNA sequence of the phage DNA could also result in the entry of the superinfecting phage DNA if the SieA protein could no longer bind to the specific DNA sequence on the phage DNA. (C) DES mutagenesis of P22 *sieA*$^{+}$ lysogen failed to produce a DES mutant that is sensitive to P22 *virB3* infection. (D) Suppressor analysis of the phage DNA. Introducing mutations in the phage DNA could potentially identify phage genes that might be involved in the transport of phage DNA across the cytoplasmic membrane of the host. Sensitivity of a P22 *sieA*$^{+}$ lysogen to mutated P22 *virB3* particles would suggest that the SieA protein is directly acting on the phage DNA or a phage-encoded protein to prevent entry of the superinfecting DNA.

A)



B)



C)

DES Mutagenesis of SieA+ Lysogen

1600 DES SieA+ lysogen mutants

↓

0 P22 virB3 sensitive colony

↓

SieA could still prevent entry of phage DNA

D)

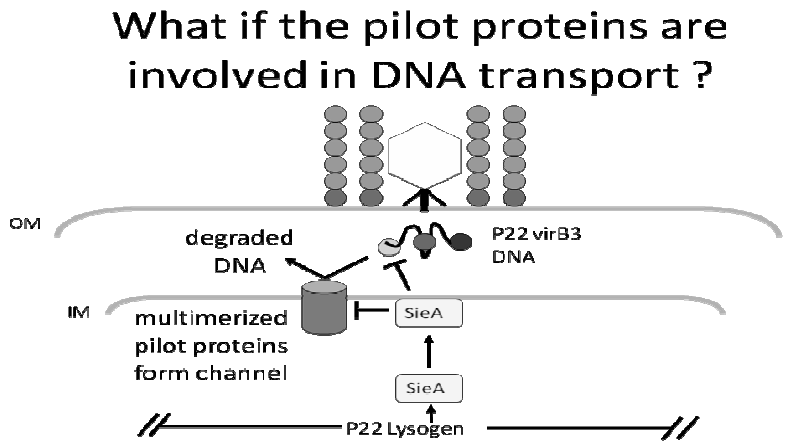


Figure 4.10: *Suppressor analysis*, Continued.

infection (Figure 4.10B). To this end, the P22 *sieA*$^{+}$ lysogen strain was mutagenized with diethylsulfate (DES) and then screened for the presence of P22-sensitive mutants by replica plating on EBU plates supplemented with lytic P22 *virB3* particles. P22 lysogen DES mutants that became P22^{S} appeared blue on the EBU plates while P22 lysogen DES mutants that remained resistant to lytic P22 appeared white. DES mutants that appeared blue (i.e. P22^{S}) on the EBU plates were picked from the corresponding master plate and cross-streaked against P22 *virB3* to confirm their P22^{S} phenotype.

DES mutagen failed to yield suppressor mutants. Although the addition of DES to P22 *sieA*$^{+}$ lysogen generated some mutants, these mutants remained resistant to P22 *virB3* infection (Figure 4.10C). This indicated that SieA could still exert its role in excluding the superinfecting DNA of P22 *virB3* from gaining access to the cytoplasm of the P22 *sieA*$^{+}$ lysogen. Since the mutagenesis by DES may not have been sufficiently extensive, it would be difficult to conclude from these experiments that no host factor is utilized by the SieA protein to exclude any incoming phage DNA.

The suppressor analysis was extended and applied to phage-encoded proteins (Figure 4.10D). Suppressor analysis of the phage DNA may provide some information whether there are phage-encoded factors that might contribute to the transport of phage DNA across the cytoplasmic membrane of the host cell. As mentioned above, the SieA protein could probably exert its exclusionary abilities by interacting with any of the pilot proteins closely associated with the superinfecting DNA. A mutation in any or all three pilot proteins could prevent SieA from interacting with the incoming phage DNA resulting in the entry of the DNA into the cytoplasm of the host cell. Aside from genes encoding the pilot proteins, other phage genes that contribute to the uptake of the phage DNA into the cytoplasm of the host might be identified by mutating the phage DNA.

The phage DNA was mutated in two ways. One strategy to mutate the phage DNA was by propagating the phage in a *mutD* strain of *Salmonella* Typhimurium. The *mutD* strain has mutations in the proofreading function of the DNA polymerase III. Phage DNA propagated in the *mutD* strain would accumulate mutations when the DNA polymerase tries to edit the replicated DNA.

The P22 *virB3* derivative of P22 was propagated in the *mutD* strain of *Salmonella* Typhimurium for suppressor analysis. P22 *virB3* has mutations in the binding site for the c2 repressor which prevents the c2 repressor expressed by a P22 lysogen host cell from inhibiting lysis by the superinfecting P22 *virB3* DNA. A P22 *virB3* DNA that can bypass the exclusionary restrictions of the SieA protein will enter the cytoplasm of a P22 lysogen and will immediately proceed to lyse the cell.

The P22 *virB3* phage lysate generated from the *mutD Salmonella* strain was titered and serially diluted. The serial dilutions of the mutated P22 *virB3* phage particles were then used to infect both MST 1757 and a P22 *sieA*$^+$ lysogen of *Salmonella* Typhimurium by adding 10 µl spots of the mutated phage lysate onto EBU plates with top agar containing the respective host cells. A P22 *sieA*$^+$ lysogen would normally be resistant to an incoming P22 *virB3* DNA because the SieA protein prevents entry of the incoming DNA by either blocking the DNA per se or by inhibiting the function of any of the pilot proteins guiding the DNA across the cytoplasmic membrane. If the mutated P22 *virB3* DNA makes it across the cytoplasmic membrane of the P22 *sieA*$^+$ lysogen (e.g. plaques are formed), then that would imply that the mutation in the P22 *virB3* DNA allowed the DNA to bypass the exclusionary function of the SieA protein.

The mutD-propagated P22 virB3 DNA failed to infect the P22 sieA$^+$ *lysogen.* The *mutD*-mutated P22 *virB3* particles were not successful in infecting the P22 *sieA*$^+$ lysogen (Table 4.2). Infecting the P22 *sieA*$^+$ lysogen host with the mutated P22 *virB3* particles did not produce any lysogen host cell that was sensitive to the incoming *virB3* DNA. Even though the mutated P22 *virB3* particles could not infect the P22 *sieA*$^+$ lysogen, they were still able to infect *Salmonella* Typhimurium MST 1757. The P22 *sieA*$^+$ lysogen showed sensitivity to phage KB1, as previously reported by Boro and Brenchley (16).

Table 4.2: Plaque formation assay of *mutD*-mutated P22 *virB3*. Sensitivity of the P22 *sieA*$^+$ lysogen to *mutD*-propagated P22 *virB3* particles was compared to MST 1757. Infecting the P22 *sieA*$^+$ lysogen host with the mutated P22 *virB3* particles did not produce any visible plaque. The P22 *sieA*$^+$ lysogen showed sensitivity to phage KB1 which was shown to lyse *sieA*$^+$ lysogens by Boro and Brenchley (16).

Strains	Phages used for forming plaques		
	P22 *virB3*	*mutD*-mutated P22 *virB3*	Wild-type KB1 (+) control
MST 1757	2.4 x 10^{11} pfu/ml	4.0 x 10^9 pfu/ml	Plaques formed
sieA$^+$ Lysogen	No plaques	No plaques (no suppressor mutants found)	Plaques formed

Another way phage DNA was mutated was by directly adding hydroxylamine (HA) into a concentrated lysate of P22 *virB3*. Mutagenesis was monitored until a survival rate of between 0.1 to 1% was reached. This took approximately 24 to 36 hr of incubating the phage particles in freshly-made hydroxylamine solution. Efficiency of hydroxylamine mutagenesis of P22 *virB3* was determined by concurrently mutagenizing wild-type P22. Wild-type P22 repeatedly produced 10 to 100 times more clear plaques after HA mutagenesis.

The hydroxylamine-mutated P22 virB3 DNA failed to infect the P22 sieA$^+$ *lysogen*. Infecting the P22 *sieA*$^+$ lysogen cells with the hydroxylamine-treated P22 *virB3* particles did not produce any suppressor mutants (Table 4.3). No hydroxylamine-treated P22 *virB3* phage particle was found to successfully lyse the P22 *sieA*$^+$ lysogen. This would mean that the SieA protein of the P22 lysogen could still block the entry of the superinfecting DNA. One possible explanation why no suppressor mutants were found might be that the SieA protein interacts with not just

one pilot protein but all three and the hydroxylamine mutagenesis was not extensive enough to mutate all three pilot proteins. Another possible explanation is that SieA partly interacts with the phage DNA while it associates with the pilot proteins. This would demand that mutagenesis be more extensive to not just mutate the genes coding for the pilot proteins but also mutate specific sequences on the phage DNA to prevent complete binding of the SieA protein to the incoming phage DNA. A third possibility is that the SieA protein interacts with a protein complex formed by both phage- and host-encoded factors and the only way to prevent SieA exclusion of the phage DNA is to modify all phage and host factors involved in the SieA-orchestrated exclusion process. This would require mutagenesis of both the phage DNA and the host chromosome at the same time. The last possibility is that the phage- or host-encoded factors interacting with the SieA protein during superinfection exclusion are encoded by essential genes.

Table 4.3: Plaque formation assay of hydroxylamine-treated P22 *virB3*. Hydroxylamine mutagenesis of P22 *virB3* did not generate any suppressor mutant. No hydroxylamine-treated phage particle was isolated that could successfully lyse the P22 *sieA*$^{+}$ lysogen.

Strains	Phages used to form plaques		
	P22 *virB3*	Hydroxylamine-treated P22 *virB3*	Wild-type KB1 (+) control
MST 1757	1.0 x 10^{11} pfu/ml	2.6 x 10^{10} pfu/ml	Plaques formed
sieA^{+} Lysogen	No plaques	No plaques (no suppressor mutants found)	Plaques formed

C. Summary of Genetic Approaches

Both transposon and chemical mutagenesis produced numerous $P22^R$ mutants with mutations in LPS biosynthetic genes that thereby blocked adsorption. One $P22^R$ mutant generated by nitrosoguanidine mutagenesis showed normal adsorption kinetics but had a deficiency in transduction. The mutation created by nitrosoguanidine did not completely eliminate the ability of the $P22^R$ mutant to transduce phage DNA. These studies suggest that either the host proteins responsible for phage DNA translocation are not absolutely required or that they are encoded by essential genes.

Extensive suppressor analyses failed to identify host and phage factors involved in P22 translocation. These studies suggest that the mechanism of SieA exclusion may not completely overlap with the mechanism of phage DNA transport. Proteins required in one mechanism may not be required in the other.

II. Biochemical Approaches

Biochemical approaches were employed to complement and validate the results obtained from the genetic approach. Biochemical strategies focus on host factor isolation at the protein level which bypasses the premature degradation and potential instability of the mRNA that might occur during *in vivo* transcription of the gene of interest. The most important benefit that could be derived from pursuing biochemical approaches is that the host factor can be isolated whether or not it is encoded by an essential gene as long as the expression host cells are grown in rich nutrient media.

A. Co-elution of host factor with the pilot proteins

The first attempt made to isolate the host factor biochemically was to co-elute the potential host factor from purified membrane proteins during the purification of the pilot proteins. An assumption was made that the potential host factor interacts with one or all of the pilot proteins during transport of the phage DNA across the cytoplasmic membrane. The possible protein-protein interaction between the host factor and any of the pilot protein would enable straight-forward and efficient isolation of the host factor by purifying the pilot protein that associates tightly with the host factor. Purification of the pilot proteins was facilitated by tagging the pilot proteins with 2 consecutive tags (6xHis and S*tag) in the N-terminus of the protein. The genes that code for the pilot proteins were cloned immediately downstream of the T7 promoter in the IPTG-inducible pET30 expression vector obtained from Novagen. IPTG induction releases the Lac inhibitor repressing the expression of the T7 gene 1 (T7 polymerase) found in the DE3 prophage located in the *E. coli* bacterial chromosome. The expressed T7 polymerase binds to the T7 promoter of the pET30 vector and transcribes the gene of interest. Membrane proteins purified from the *Salmonella* Typhimurium membrane extract were added to the nickel-bound pilot proteins after the DE3 *E. coli* cleared lysates have flowed through the nickel-charged column and after the column has been washed three times with a wash buffer.

Over 90% purity of the pilot proteins was obtained by performing two consecutive affinity chromatography purification procedures. The first purification was performed using a nickel column that specifically binds to the hexahistidyl (6xHis) tag of the fusion protein. The second column was packed with an S*tag agarose resin that has a strong affinity to the S*tag of the fusion protein. Each purification step was

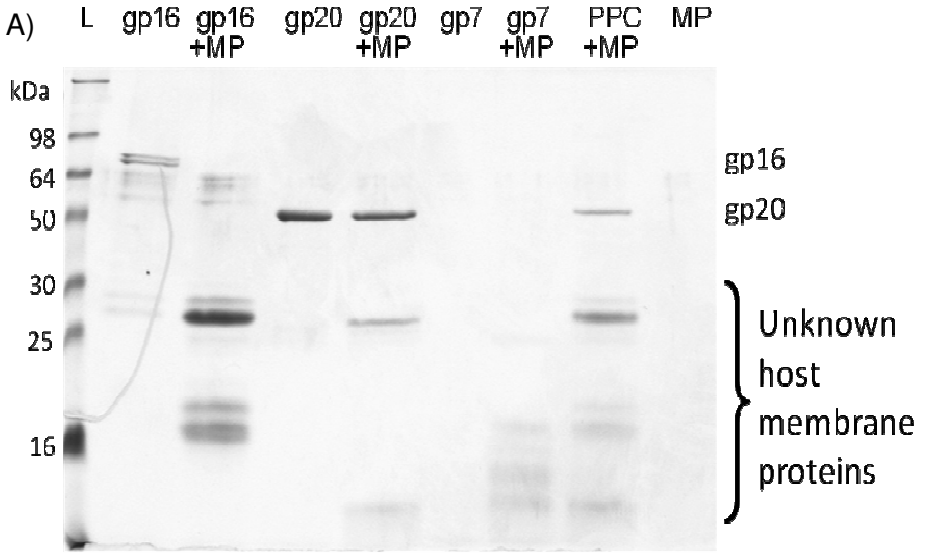
followed by a buffer exchange protocol using a centricon filter column to remove any residual imidazole or magnesium chloride used in the elution of the protein from the respective affinity columns. *Salmonella* Typhimurium membrane proteins that co-eluted with the purified pilot protein were visualized on an SDS polyacrylamide gel that was stained with Coomassie blue.

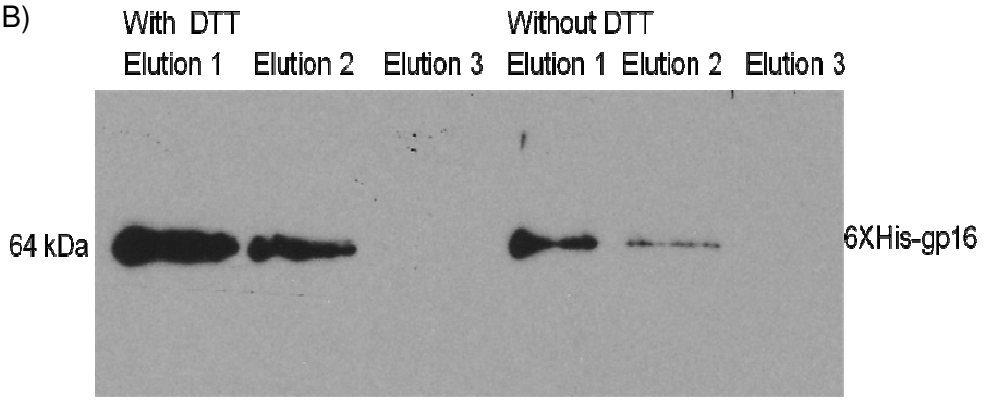
*Host membrane proteins co-elute with 6xHis-S*tagged pilot proteins.* Figure 4.11A shows some of the membrane proteins from *Salmonella* Typhimurium that co-eluted with 6xHis-S*tag-gp16 after the dual affinity column purification procedures. These co-eluted membrane proteins are all excellent candidates for the potential host factor involved in phage DNA transport since they remained bound to the pilot protein after stringent washes in each affinity column and after two centricon filtration steps to remove residual elution reagents. Although all these co-eluted proteins specifically bound the gp16 protein, most may prove to be “accidental” partners of gp16; they probably bound somewhere else on the gp16 protein distinct from the binding site for the host factor involved in phage DNA transport. To eliminate some of the co-eluted membrane proteins as potential host factors in the uptake of phage DNA, the use of cross-linking compounds was investigated.

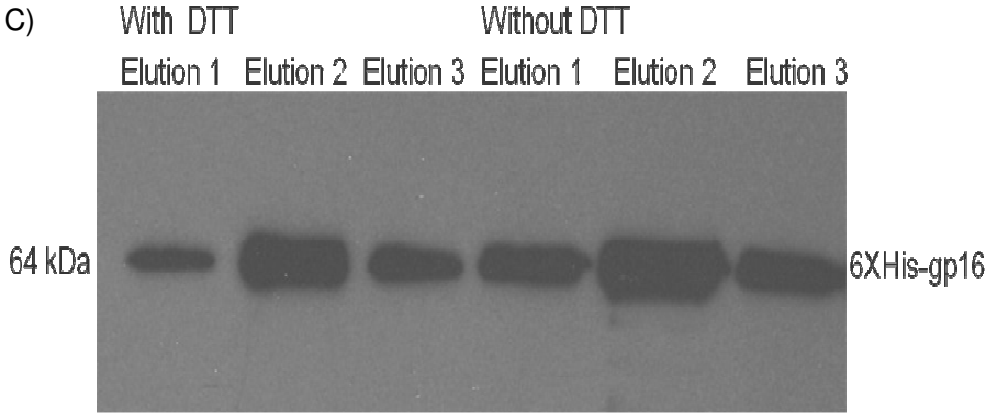
B. Cross-linking of host factors to 6xHis-gp16

The use of cross-linkers was investigated to narrow down the host membrane proteins that co-eluted with the purified pilot proteins. Cross-linkers are useful in stabilizing protein-protein interactions or protein-DNA interactions. The use of cross-linkers may prove beneficial in identifying the correct host factor since it could increase the contrast between the “true” protein-protein interactions and the

Figure 4.11: *Biochemical approaches used to isolate host factors involved in phage DNA transport.* (A) Host membrane proteins co-elute with 6xHis-S*tagged pilot proteins. Coomassie blue stain of a 12% SDS gel showing co-eluted membrane proteins from *Salmonella* Typhimurium after dual affinity column purification of the pilot proteins. L = Protein Ladder, MP = Membrane Proteins, PPC = Pilot Protein Complex. Purified membrane proteins were passed through the S*tag agarose column to rule out any potential binding of the membrane proteins to the S*tag resin (last lane). (B) Cross-linking 6xHis-gp16 to membrane proteins using Dithiobis-succinimidylpropionate (DSP). *Salmonella* membrane proteins were added to nickel-bound 6xHis-gp16 and cross-linked using DSP. Samples were not boiled but were either treated or not treated with 50 mM DTT (37^{o}C for 30 min) prior to loading on a 12% SDS gel. 6xHis-gp16 was detected by Western blotting with rabbit anti-6xHis. (C) Cross-linking *cytoplasmic membrane* proteins to the 6xHis-gp16 protein using DSP. Sarkosyl-extracted *Salmonella* cytoplasmic membrane proteins were added to nickel-bound 6xHis-gp16 and cross-linked using DSP. Samples were not boiled but were either treated or not treated with 50 mM DTT (37^{o}C for 30 min) prior to loading on a 12% SDS gel. 6xHis-gp16 was detected by Western blotting with rabbit anti-6xHis. (D) *In vivo* cross-linking of gp16-6xHis to host proteins using Dimethyl Suberimidate•2 HCl (DMS). gp16-6xHis was expressed and targeted to the periplasmic space of the *Salmonella* Typhimurium expression host using the Novagen pET26 vector. The gp16-6xHis was either cross-linked using DMS to host proteins or not cross-linked and then purified using the nickel column. Elutions were ran on a 12% SDS gel and transferred to a PVDF membrane. gp16-6xHis was detected using the rabbit anti-6xHis antibody. (E) Virus Overlay Protein Blot Assay (VOPBA) of *Salmonella* membrane proteins reveals a 40 kDa membrane protein that specifically bound the gp16 pilot protein. Membrane proteins were ran on a 10% SDS gel and transferred to a PVDF membrane. The membrane proteins were denatured in 6M guanidine-HCl and then renatured by washing the PVDF membrane with decreasing concentration of guanidine-HCl. The membrane was cut into strips and each strip was incubated with a specific twice-purified pilot protein prior to Western blotting with the rabbit anti-6xHis antibody to detect the specific membrane protein bound by the 6xHis-tagged pilot protein. The first lane shows that the membrane proteins do not cross-react with the rabbit anti-6xHis antibody.


A)
L
gp16
gp16 +MP
gp20
gp20 +MP
gp7
gp7 +MP
PPC +MP
MP
kDa
98
64
50
30
25
16
gp16
gp20
Unknown host membrane proteins


B)
With DTT
Without DTT
Elution 1
Elution 2
Elution 3
Elution 1
Elution 2
Elution 3
64 kDa
6XHis-gp16


C)
With DTT
Without DTT
Elution 1
Elution 2
Elution 3
Elution 1
Elution 2
Elution 3
64 kDa
6XHis-gp16

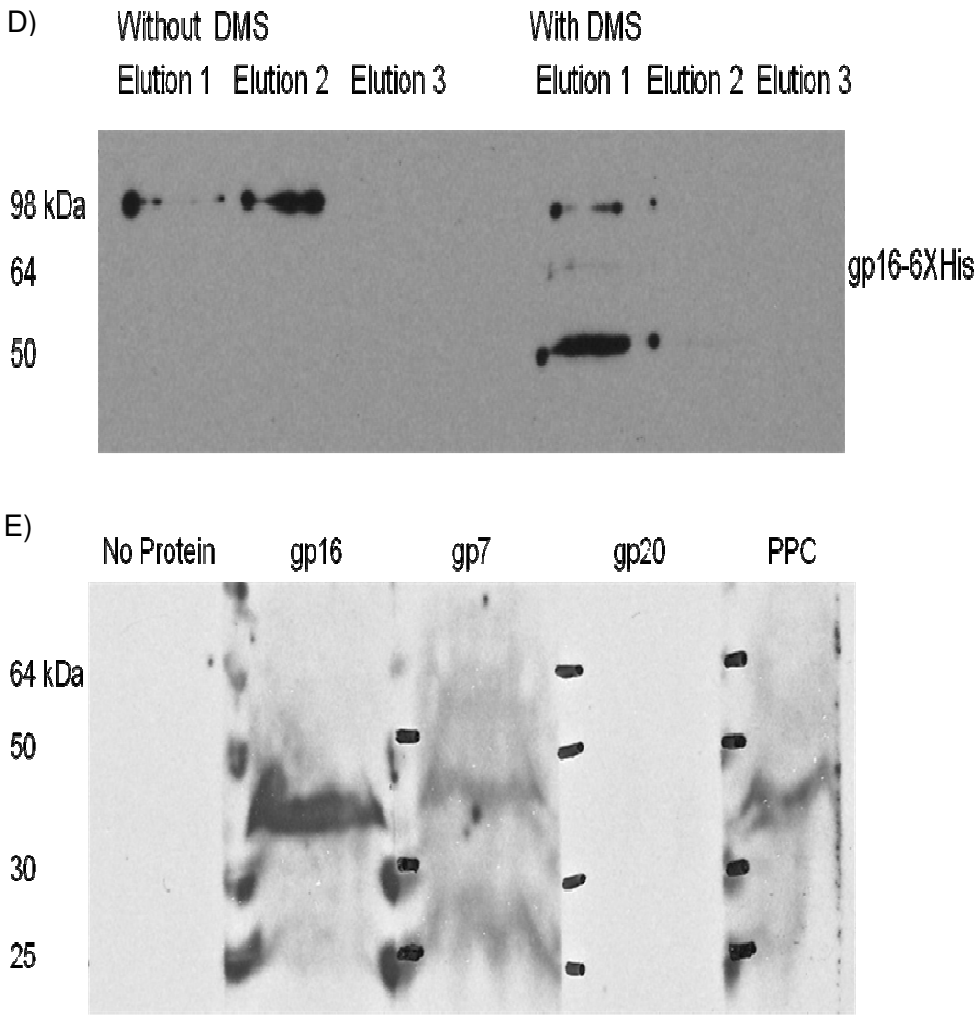


Figure 4.11: *Biochemical approaches used to isolate host factors involved in phage DNA transport,* Continued.

"accidental" protein-protein interactions described above or that might have resulted from molecular mimicry. It is not uncommon for proteins to have similar shapes or have short amino acid sequences that are almost identical. Proteins with domains or segments that slightly differ from the host factor in a few amino acids could potentially bind the pilot proteins. Employing the use of cross-linkers would not only confirm the results obtained from the previous co-elution

experiment but could also reveal more protein-protein interactions that were not established, and therefore not seen, in the co-elution experiment. Testing different cross-linkers was necessary since each one has a specified spacer arm length that will covalently link two proteins permanently.

Purified *Salmonella* Typhimurium membrane proteins were used as the source of the potential host factor instead of the entire host proteins to avoid unnecessary exposure of the pilot proteins to soluble cytoplasmic proteins of the host. The soluble cytoplasmic host proteins are proteins which would not normally be in the vicinity of the pilot proteins during phage DNA transport. “Accidental” protein-protein interactions with the pilot proteins are minimized by excluding the soluble cytoplasmic host proteins.

The cross-linkers tested were Dimethyl Suberimidate•2 HCl (DMS) and Dithiobis-succinimidylpropionate (DSP). Dimethyl suberimidate (DMS) is a water soluble, membrane permeable, homobifunctional imidoester cross-linker with a spacer arm length of 1.1 nm. The imidoester functional group is one of the most specific acylating groups available for the modification of primary amines and has minimal cross-reactivity toward other nucleophilic groups in proteins (115). In addition, the imidoamide reaction product does not alter the overall charge of the protein, potentially retaining the native conformation and activity of the protein. Unlike DMS, the reaction with DSP is reversible and can be cleaved with 50-150 mM dithiothreitol (DTT) at 37^{o}C for 30 min. The presence of DTT will reduce the disulfide bond found in the internal molecular structure of DSP.

DSP failed to cross-link 6xHis-gp16 to any host membrane protein. Figure 4.11B shows the result of using DSP to cross-link 6xHis-gp16 to host membrane proteins. No other bands were detected aside from the 6xHis-gp16 protein (64 kDa band) in the absence of DTT which indicates that no membrane protein was cross-linked to the gp16 protein.

To enrich for cytoplasmic membrane proteins, *Salmonella* Typhimurium membrane extract was solubilized with 2% sarkosyl. The purified cytoplasmic membrane proteins were cross-linked to the 6xHis-gp16 protein using DSP. Figure 4.11C shows the result of using DSP to cross-link purified cytoplasmic membrane proteins to the 6xHis-gp16 protein. Once again, there were no other bands detected aside from the 6xHis-gp16 band in the absence of DTT which suggests that there were no cytoplasmic membrane proteins cross-linked to the 6xHis-gp16 protein.

DMS cross-linked gp16-6xHis to host proteins in vivo. *In vitro* cross-linking of gp16 to purified host membrane proteins was not successful probably because the gp16 protein and the host membrane proteins were somehow altered during the purification process. *In vivo* cross-linking was attempted to circumvent the issue of working with altered proteins. gp16-6xHis was expressed and targeted to the periplasmic space of the *Salmonella* Typhimurium expression host cell using the Novagen pET26 vector. After IPTG-induced expression, gp16-6xHis was either cross-linked to host proteins using DMS or not cross-linked and then purified using nickel-charged columns. Elutions were ran on a 12% SDS gel and transferred to a PVDF membrane. gp16-6xHis was detected using the rabbit anti-6xHis antibody. Figure 4.11D shows the result of the *in vivo* DMS cross-linking experiment. Most, if

not all, of the gp16-6xHis proteins were already bound to another protein (98 kDa band) prior to DMS cross-linking and this protein-protein interaction was resistant to SDS. Purified gp16 normally runs as a 64 kDa protein and it never shows up as a 98 kDa protein band. Targeting the gp16 protein to the periplasmic space of the host cell allowed it to find the host protein that it interacts with *in vivo* during the normal process of P22 infection of the host cell. Addition of DMS produced another gp16-containing protein complex that ran at around 50 kDa. This could either be a more compact globular complex of homomultimeric gp16 proteins or a mixture of the gp16 protein bound to the host factor. The faster migration into the gel may be due to multiple permanent cross-links made by DMS which could not be reversed by SDS. This data suggests that there is a host protein having a molecular weight of approximately 34 kDa that might be interacting with the gp16 pilot protein during P22 DNA transport across the cytoplasmic membrane of *Salmonella* Typhimurium.

C. Isolating host factors using Virus Overlay Protein Blot Assay (VOPBA)

Another biochemical approach was attempted to confirm the results obtained in the *in vivo* cross-linking experiment using DMS. A protein hybridization technique called Virus Overlay Protein Blot Assay (VOPBA) was used to isolate potential membrane proteins that could form stable interactions with the pilot proteins. The VOPBA technique has been shown to identify several virus receptors including those for adenoviruses, arenaviruses and coronaviruses (93).

VOPBA isolated a 40 kDa membrane protein that specifically associates with the 6xHis-gp16 and 6xHis-gp7 pilot proteins. The VOPBA PVDF membrane shows that there is a 40 kDa membrane protein that specifically associates with the 6xHis-gp16 pilot protein (Figure 4.11E). 6xHis-gp7 was found to bind a 40 kDa

protein and a slightly smaller membrane protein. 6xHis-gp20 did not associate with any of the membrane proteins and the PPC blot reflected the same result as the gp16 blot. The associations detected between the gp16 and gp7 pilot proteins and the membrane proteins are specific since the membrane proteins did not cross react with the rabbit anti-6xHis antibody. The only time a membrane protein produced a chemiluminescent signal was when it was pre-incubated with one of the pilot proteins. The VOPBA data confirmed the presence of a host protein that might be involved in the transport of phage P22 DNA across the cytoplasmic membrane of the host.

Discussion

Transposon mutagenesis failed to identify a host factor that might be involved in the translocation of P22 DNA into the host cytoplasm. A total of 16,650 transposon mutants were screened and 1,018 were found to be resistant to P22 infection. Out of these $P22^R$ mutants, only 118 were resistant to phage Ffm and only 11 out of these 118 were sensitive to phage Felix-O1. The LPS profile of the remaining 11 mutants on an SDS gel revealed that they had a mutation in the *rfc* gene.

Chemical mutagenesis by nitrosoguanidine (MNNG) proved to be more effective in generating a $P22^R$ mutant with a defect in transduction. MNNG may have been more effective than any of the transposons used in generating $P22^R$ mutants because it was able to target multiple genes whose gene products are involved in phage DNA transport. Chemical mutagenesis can also produce missense mutants rather than strictly null mutants as commonly occur with

transposon mutagenesis. Alternatively, it is also possible that the transposon mutagenesis was not extensive enough to produce the desired number of $P22^R$ mutants. This might very well be the case since it required a starting number of at least 3,600 $P22^R$ mutants for the MNNG mutagenesis to come up with 5 mutants with normal adsorption kinetics. The total consolidated number of $P22^R$ mutants from all transposon mutagenesis was only 1,018. This number may not have been statistically sufficient to produce the desired number of $P22^R$ mutants with normal adsorption kinetics after undergoing all the screening processes.

Suppressor analysis was unsuccessful in identifying both host and phage factors involved in P22 DNA translocation. DES mutagenesis of P22 *sieA*$^+$ lysogen failed to produce mutant cells that are sensitive to lytic P22 *virB3* which suggests that there are no host factors involved in the exclusion of superinfecting DNA by the SieA protein. This would imply that the mechanism of superinfection exclusion does not entirely overlap with the mechanism of phage DNA transport. The SieA protein could be interacting directly with the pilot proteins coating the superinfecting DNA to exert its influence without the need for a host protein.

Mutagenesis of the phage DNA by propagating the phage DNA in the *mutD* strain of *Salmonella* or by directly adding hydroxylamine to the phage lysate also failed to produce suppressor mutants. The mutated phage particles were unable to bypass the hindrance presented by the SieA protein. This suggests that either phage factors are not involved in the mechanism of superinfection exclusion or the mutagenesis was not extensive enough to permit the phage factors to pass through the cytoplasmic membrane undetected by the SieA protein. Since the methods used to mutagenize the phage DNA rarely result in major alterations in

the translated proteins, it would be hard to imagine how the SieA protein would fail to detect the phage factors involved in the exclusion of the phage DNA. In spite of the minor point mutations in the phage DNA, the overall shape of the phage proteins was maintained. They were adequately recognized and therefore excluded by the SieA protein.

Although extensive nitrosoguanidine mutagenesis identified a $P22^{R}$ mutant with a defect in transduction, the deficiency in transduction was incomplete. The integration of all the genetic studies suggests that either host proteins are not absolutely required or that host proteins responsible for phage DNA translocation are encoded by essential genes. Biochemical approaches were therefore explored to confirm the results obtained from the genetic studies and to extend the isolation of host factors to proteins encoded by essential genes.

Results from the co-purification experiment revealed that there are host membrane proteins that co-elute with the pilot proteins after two consecutive affinity column purification procedures. The binding of these membrane proteins to the pilot proteins is highly specific since they remained bound to the pilot proteins after a series of stringent washes and buffer exchanges. This suggests that the pilot proteins coating the phage DNA can interact with multiple host membrane proteins. Such an interaction can result in a formation of a complex of host membrane proteins linked together to channel the phage DNA across the cytoplasmic membrane.

Targeting the gp16-6xHis proteins to the periplasmic space of *Salmonella* Typhimurium using pET26 revealed that there is a host protein that interacts with

the gp16 protein during the normal process of P22 infection of the host cell. Most, if not all, of the gp16-6xHis proteins were found already bound to another protein (98 kDa band) prior to DMS cross-linking and this protein-protein interaction was resistant to SDS. Since purified gp16 normally runs as a 64 kDa protein in an SDS polyacrylamide gel, this would suggest that the host protein bound to the gp16-6xHis protein has a molecular weight of approximately 34 kDa.

The Virus Overlay Protein Blot Assay (VOPBA) proved to be successful in isolating host membrane proteins that interact specifically with the 6xHis-gp16 and the 6xHis-gp7 pilot proteins. One of the host membrane proteins isolated by VOPBA has a molecular weight of approximately 40 kDa. The interaction between the ~40 kDa membrane protein and the pilot proteins is specific since no membrane protein was found to cross react with the rabbit anti-6xHis antibody.

Summary

The combined application of genetics and biochemistry proved to be beneficial in the isolation of the host factors involved in P22 DNA transport. The isolation of a $P22^{R}$ mutant that had a deficiency in transduction using nitrosoguanidine suggests that there are host factors available for P22 DNA transport across the cytoplasmic membrane. The fact that the transduction deficiency is incomplete indicates that the P22 DNA can cross the cytoplasmic membrane without the need for any host factor. This finding generated by genetic analysis confirms the results provided in the earlier chapters that the phage-encoded pilot proteins are responsible for the uptake of P22 DNA into the host cytoplasm and that the energy fueling the transport comes from the host.

The VOPBA-isolated host membrane protein that interacted with gp16 and gp7 still needs to be identified. Knocking out the gene that codes for this host membrane protein would prove whether or not it is involved in the uptake of P22 DNA. This would also confirm whether P22 requires a host factor in transporting its DNA across the cytoplasmic membrane of the host.

Chapter V. Summary

The overall goal of this thesis was to elucidate the mechanism of P22 DNA transport across the cytoplasmic membrane of *Salmonella* Typhimurium. It was not only important to ascertain if there were host factors involved in the uptake of phage DNA but also necessary to investigate if phage-encoded factors were sufficient for transporting the phage DNA across the host cytoplasmic membrane. Understanding the active transport of a macromolecule across a barrier presented by the cytoplasmic membrane provided the impetus to study the bioenergetics involved in phage DNA transport.

Relevant findings in the preceding chapters will be summarized in this chapter followed by a discussion on how these findings relate to one another. This chapter concludes by presenting some future studies which might be of benefit to anyone who would be interested in pursuing this area of research. This thesis revealed new insights into the biology of phage DNA transport, but also generated more questions. This goes to show that the interacting field of phage biology and membrane biochemistry is far from being well understood.

Proteins that constitute the transporter apparatus for P22 DNA uptake into the host cytoplasm are phage-encoded (Chapter II). The formation of the Pilot Protein Complex (PPC), the membrane-disrupting activity of gp16 and the ability of the pilot proteins to transport DNA into liposomes suggest that the factors required for transporter apparatus assembly are phage-encoded.

The interaction between all three pilot proteins not only makes it easier and faster to bind to nonspecific areas of the phage DNA but also makes the binding to

the DNA stronger and more stable to withstand the constant intrusion and interference of the nucleases in the periplasmic space. In addition, assembly of the transporter apparatus is made more efficient since all the components that constitute the channel are already in close proximity to one another. The phage DNA is also not far behind since the combined efforts of all the DNA-binding pilot proteins brings the DNA right next to or possibly into the pore formed by the pilot proteins.

Leading the “pack” is the gp16 protein which must not only target the phage DNA to the cytoplasmic membrane but must also anchor it there by embedding itself into the hydrophopic core of the cytoplasmic membrane. How the gp16 protein changes conformation prior to and immediately after membrane association would certainly shed some light into how channel formation is initiated by this protein. Pore formation by the gp16 protein was demonstrated by its ability to release the fluorescent dye calcein from inside liposomes. Since the release of a 0.67 nm calcein molecule (82) from inside liposomes could not provide adequate information whether the pore size is wide enough to accommodate the uptake of bigger molecules like DNA (2 nm in diameter) (55) or whether the pilot proteins are required for DNA translocation, we had to test the actual transport of DNA into liposomes. The liposomal DNA transport assay not only revealed that the pilot proteins are required for DNA transport into liposomes but that DNA uptake is also dependent on the presence of a membrane potential.

The gp16-dependent transport of radiolabeled DNA inside liposomes with an artificially-created membrane potential provides convincing evidence that the minimum proteins required to form the transporter apparatus are phage-encoded. The requirement for gp7 and gp20 in P22 infection may not only be due to their role in

protecting the phage DNA from nucleases in the periplasmic space. They might also have some sort of a “chaperone” function which enables them to keep the DNA in a specific conformation conducive for facilitated translocation by the transporter apparatus.

The participation of host membrane proteins as transporter molecules still remains a distinct possibility. Neal *et al.* (123) showed that P22 can transduce *E. coli* harboring a cosmid that carries the *Salmonella* Typhimurium *rfb* cluster and *rfc* gene to constitutively express the O-antigen residues required for P22 adsorption. The ability of P22 to infect a strain of *E. coli* expressing the appropriate LPS structure either supports the idea that the transporter apparatus is composed of phage-encoded proteins or that the transporter molecule is found in both *E. coli* and *Salmonella* Typhimurium. It would be interesting to see if P22 can also transport its DNA across the cytoplasmic membrane of other Gram negative bacteria if these bacterial species are allowed to express the *Salmonella* LPS on their outer membrane. That would lend more support to the idea that uptake of P22 DNA into the host cytoplasm is dependent on the phage-encoded pilot proteins.

The membrane potential of the host is required for phage P22 DNA uptake (Chapter III). The transport of DNA across a hydrophobic membrane requires energy. Testing different compounds known to inhibit specific sources of energy inside a bacterial cell revealed that it is the membrane potential across the cytoplasmic membrane of the host that supplies most, if not all, of the energy required for P22 DNA transport. Valinomycin, which specifically dissipates the membrane potential without affecting the proton gradient, reduced transduction efficiency by 63%. The combination of valinomycin and arsenate did not produce additional

reduction in transduction efficiency which suggests that the energy from ATP hydrolysis does not contribute in powering the translocation of the phage DNA into the host cytoplasm. Nigericin, which dissipates the proton gradient but not the membrane potential, had no effect on transduction efficiency. All together, these results provide convincing evidence that the major energy source for P22 DNA transport is provided by the membrane potential of the host cell.

The membrane potential across the cytoplasmic membrane of the host cell is a component of the proton motive force generated by proteins involved in the electron transport chain which pumps out protons from the cytoplasm of the cell. Proteins involved in the electron transport chain are highly conserved and are essential for host survival since the process of oxidative phosphorylation generates the majority of the ATP molecules needed for active metabolism. Hence, a mutation in genes coding for proteins involved in the electron transport chain might be detrimental to the host. This is worth mentioning because one of the $P22^{R}$ mutants isolated using nitrosoguanidine had a deficiency in transduction and the point mutations may have been in proteins directly involved in the electron transport chain. This would probably explain why a transduction mutant with a 100% defect in transduction could not be isolated. Only minor point mutations in genes coding for proteins involved in the electron transport chain can be tolerated. The degeneracy and redundancy of the amino acids may also have played a key role in averting lethal point mutations which consequently maintained the overall functional shape and charge of the essential gene products.

Factors for the uptake of phage P22 DNA into the cytoplasm of Salmonella Typhimurium are host-encoded (Chapter IV). Attempts were made to

isolate host proteins that might be directly or indirectly involved in both the mechanism of phage DNA transport and the mechanism of superinfection exclusion. Although nitrosoguanidine mutagenesis was successful in generating an adsorbing $P22^R$ mutant with a defect in transduction, the deficiency in transduction was incomplete. It can still transduce 30% as efficiently as *Salmonella* MST 1757 cells. Together with results from the rest of the direct genetic approaches, this suggests that either host proteins are not absolutely required or that host proteins responsible for P22 DNA translocation are encoded by essential genes. Host proteins may not be absolutely required for the assembly of the transporter apparatus but they are indispensible for the generation of the membrane potential across the cytoplasmic membrane. Some of the essential host proteins that are indirectly involved in phage DNA transport are components of the electron transport chain.

Other host proteins may have been isolated using a variety of biochemical means. Targeting the gp16 protein into the periplasmic space of the host cell proved critical in isolating a host protein (~34 kDa) that may be directly involved in phage DNA translocation since the host protein interacted strongly with the gp16 protein. Targeting the gp16 protein into the periplasmic space of the host cell may have been critical since this is the exact location where gp16 would normally encounter its cognate partner during P22 infection of the host. The successful encounter and association between gp16 and the host protein may also have occurred because both proteins were in their proper *in vivo* conformation. “Fishing out” the potential host transporter proteins from purified membrane proteins using purified gp16 was unsuccessful probably because the purification steps altered the conformation of gp16, the host transporter, or both proteins. Slight modifications in the binding sites of

the cognate proteins may have weakened their interaction for each other which could not be stabilized by the addition of the DSP cross-linker.

VOPBA also isolated a host membrane protein that interacted strongly with the gp16 protein. The VOPBA protocol required denaturation and gradual renaturation of the membrane proteins which may have permitted binding of the purified gp16 protein to its cognate partner more effectively. Also, since the membrane proteins were fixed on a PVDF membrane, it was less difficult for the gp16 protein to find and bind its cognate partner.

Mechanism of phage P22 DNA transport. Irreversible binding of the gp26 tail fiber to the outer membrane of the host triggers the release and ejection of the phage DNA together with the phage-encoded pilot proteins into the periplasmic space (Figure 5.1a). The gp7 and gp20 pilot proteins ensure that the phage DNA does not come in contact with any nucleases in the periplasm while the gp16 proteins make their way to the surface of the cytoplasmic membrane. Gp16 targets and anchors the phage DNA to the cytoplasmic membrane with the help of the other pilot proteins (Figure 5.1b). Once associated with the cytoplasmic membrane, gp16 changes conformation to traverse the entire lipid bilayer and initiates channel formation (Figure 5.1c). Closely associated with the gp16 protein during channel formation are the other pilot proteins which are actively chaperoning the phage DNA to keep it in the proper conformation for uncomplicated translocation through the transporter apparatus. Assuming that the respiratory proteins encoded by essential host genes have generated and can maintain the minimum required threshold membrane potential across the cytoplasmic membrane, then phage DNA transport can commence. The membrane potential of the host is absolutely necessary to power the uptake of phage

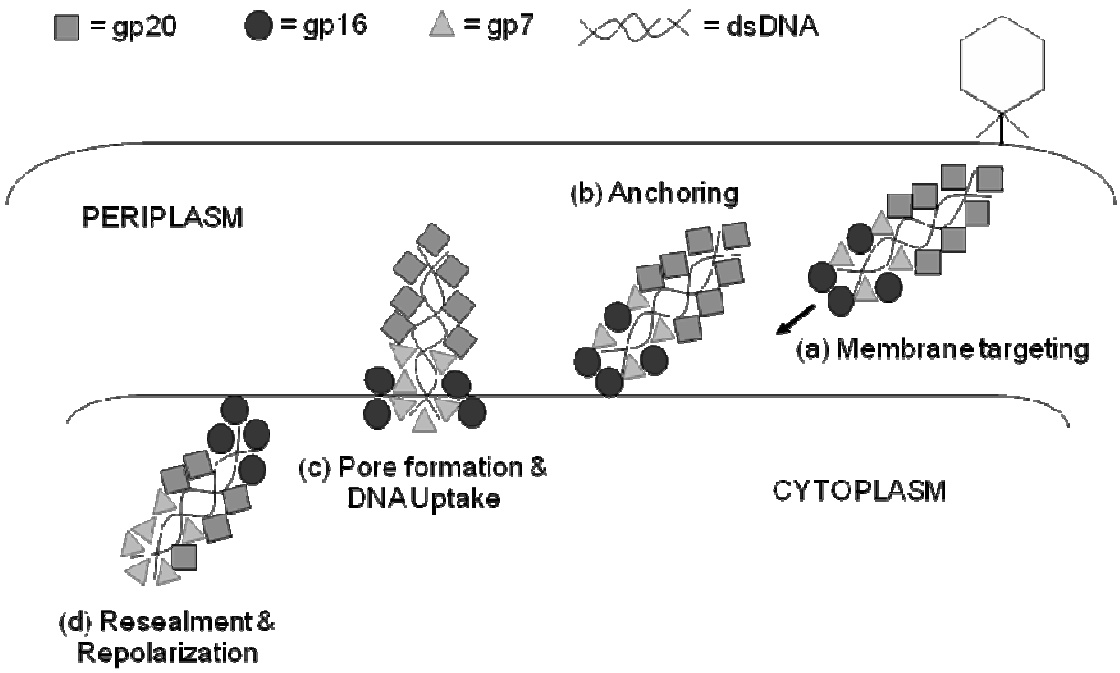


Figure 5.1: *Model of phage P22 DNA transport.* In this proposed model of phage P22 DNA translocation, phage-encoded pilot proteins form the channel for DNA transport while host respiratory proteins indirectly participate in the transport of phage DNA by maintaining a healthy membrane potential. Thus, both phage- and host-encoded factors are required for phage P22 DNA uptake.

DNA across the membrane. Without this source of energy, the phage DNA cannot be "sucked into" the host cell and it will be degraded in the periplasmic space. Being pulled in by the charge differential across the cytoplasmic membrane, the partially-unfolded gp7 and gp20 proteins still surrounding the phage DNA can now slide through the channel allowing the phage DNA to enter the cytoplasm of the host. The cytoplasmic domain of the channel-forming gp16 protein latches on to the last remaining segment of phage DNA traversing the pore to pull itself into the cytoplasm (Figure 5.1d). In the process, the pore is resealed and the cytoplasmic membrane is repolarized. Once in the cytoplasm, the DNA-bound gp16 protein can reestablish

contact with the other end of the linear phage DNA to promote recircularization. In this proposed model of phage DNA translocation, phage-encoded proteins form the channel for DNA transport while host respiratory proteins indirectly participate in the transport of phage DNA by maintaining a healthy membrane potential. Thus, both phage- and host-encoded factors are required for phage DNA uptake.

Future studies. There were questions about the mechanism of phage DNA transport that were addressed in this thesis but some of the evidence and data collected pointed to and generated more relevant questions. This section of the thesis elaborates on some of these relevant questions and provides possible experimental designs that can be implemented to answer them. In some cases, improvements on the techniques or assays performed have been outlined.

A. Genetic Strategies

1) One of the reasons why nitrosoguanidine was successful in generating the desired $P22^R$ mutant was because a large number of $P22^R$ mutants was screened to eliminate LPS mutants and to look for specific mutations in the transduction stage of P22 infection. Compared to the nitrosoguanidine mutagenesis, the total number of transposon-generated $P22^R$ mutants was less than one third of the number of $P22^R$ mutants produced by nitrosoguanidine. Creating an adequate number of transposon mutants might eventually result in a $P22^R$ mutant with normal adsorption kinetics but with a defect in transduction. Using the T-POP transposon cloned in the pRL27 plasmid carrying the *ori* R6K might also improve the outcome of the transposon mutagenesis. This will take advantage of both the potential of the T-POP transposon to transcribe essential genes downstream of the insertion site and also lose the

transposase immediately after the transposition event since the pRL27 vector can only replicate in *pir*$^{+}$ host cells.

2) Based on results of the direct genetic approaches, nitrosoguanidine (MNNG) and not diethylsulfate (DES) produced a P22^{R} mutant with the desired transduction mutation. MNNG might also prove to be more efficient than DES in creating suppressor mutants that might help isolate a host protein involved not only in superinfection exclusion but also in phage DNA transport. For these reasons, the use of MNNG to mutagenize P22 *sieA*$^{+}$ lysogen for suppressor analysis would be worth investigating.

3) Deficiency in transduction of mutant #27 (MST 5193) may be due to mutations in essential genes. Nitrosoguanidine may have introduced point mutations in genes coding for proteins that are part of the electron transport chain which makes these genes essential for host survival. Repairing the mutation in the transduction mutant (mutant #27) by complementing it with a wild-type copy of the genes that code for subunits I and II of cytochrome oxidase by transduction will confirm whether the point mutation in the transduction mutant is in genes involved in the electron transport chain. Of all the respiratory proteins involved in the electron transport chain, cytochrome oxidase is the best candidate to complement the transduction mutant because it is a “true” proton pump and it is the final transmembrane element in the respiratory chain which can accept electrons from *any* ubiquinone protein reduced by preceding respiratory enzymes in the electron transport chain. Complementing the transduction mutant with any of the preceding respiratory proteins would have less probability of success since the mutation in the transduction mutant might happen to be in genes coding for the latter respiratory proteins.

B. Biochemical Studies

1) Aside from using the pilot proteins to biochemically isolate host factors, the SieA protein can be used as the bait to “fish out” host membrane proteins by co-elution, cross-linking or VOPBA. Any host membrane protein that gets isolated by the SieA protein may be involved not only in superinfection exclusion but also in phage DNA transport. A direct comparison of the different host proteins isolated using the 6xHis-tagged SieA protein can be made with the various host proteins isolated using the 6xHis-tagged gp16 protein to detect common host proteins that are interacting with both SieA and gp16.

2) Aside from the cross-linkers described and used in this thesis, there are plenty more cross-linkers waiting to be tested and explored. One cross-linker that might be useful would be N-Succinimidyl-6-[4´-azido-2´-nitrophenylamino] hexanoate (SANPAH). SANPAH is a heterobifunctional photoactivatable imidoester cross-linker. One of the bifunctional arms of the cross-linker can be cross-linked to gp16 or SieA first by photoactivation prior to adding the SANPAH-conjugated protein to a pool of host membrane proteins. The second imidoester functional arm of SANPAH can then react with any primary amine in the pool of host membrane proteins. The advantage of hooking up the cross-linker to gp16 or SieA first is that the cross-linker is not diluted out or allowed to react with other proteins other than gp16 or SieA.

3) Are the host membrane proteins that interacted strongly with gp16 and gp7 during VOPBA isolation directly involved in phage DNA transport and/or SieA-mediated exclusion of incoming phage DNA? This is a relevant question to ask since demonstrated interaction between gp16 and any host membrane protein does not

automatically mean that the host protein is involved in the actual transport of DNA. The interaction between any host protein to either gp16 or gp7 must be followed by a DNA transport assay to show involvement of the host protein in DNA transport. Involvement in DNA transport can be demonstrated using liposomes and radiolabeled DNA as was described and performed in this thesis.

4) It would be informative to follow in more detail the changes in conformation that occurs in gp16 before and after membrane association. Deciphering the changes in conformation that occur before and after gp16 associates with the membrane would provide clues as to how the transporter apparatus is formed. Truncation studies performed on the gp16 protein revealed that the domain of gp16 corresponding to amino acid 301 to 475 is critical for membrane association or is required for the proper folding of the gp16 protein to allow membrane association. Amino acids 301 to 475 might be involved in the change of conformation of gp16 when it partitions to the cytoplasmic membrane of the host. Testing the different gp16 constructs for DNA transport into liposomes might also provide hints as to which domain of gp16 is required for DNA uptake. Introducing site-directed point mutations would narrow down which amino acid of gp16 is absolutely required for membrane association, DNA uptake or both. More structural information about the appearance of the channel can also be gained by performing crystallographic studies.

Appendix 1: List of Strains

Strains	Genotype	Reference[a]
NovaBlue E. coli cells	*endA1 hsdR17* (r_{K12}^- m_{K12}^+) *supE44 thi-1 recA1 gyrA96 relA1 lac* F'[*proA*$^+$*B*$^+$ *lacI*^{q}*lacZdelM15*::Tn*10*] (Tet^R)	Novagen
BL21 cells	F$^-$ *ompT hsdS*$_B$ (r_B^- m_B^-) *gal dcm* (DE3)	Novagen
EM 2328	NovaBlue/pET30(gene16); (Kan^R)	
EM2329	NovaBlue/pET30(gene7); (Kan^R)	
EM2330	NovaBlue/pET30(gene20); (Kan^R)	
EM1252 (DH5α)	*endA1 hsdR17* (r- m+) *supE44 thi-1 recA1 gyrA* (Nal^R) *relA1* DEL(*lacZYA-argF*)*U169* deo^R [phi80 DEL(*lacZ*)*M15*]; alias DH5α	C. Miller
EM2331	DH5α/pCDF(gene16+GST adapter+gene7); (Spec^R)	
EM2332	DH5α/pET30(gene20+GST adapter+gene7); (Kan^R)	
EM2333	DH5α/pET30(gene16+GST adapter+gene7); (Kan^R)	
EM2334	DH5α/ pCDF(gene20) S*tag removed by *Bsu36*I and *Xho*I (Spec^R)	
MST1757	*leuA414*(Am) Fels2$^-$; alias MS1363	M.Susskind
MST3663	LT2 r$^-$m$^+$ (LT,SA) *leuA414*(Am) Fels2$^-$ *endA*/pBR328 (Amp^R, Cam^R, Tet^R)	M. Lawes
MST1120	*srl::Tn10*	S. Maloy
MST2002	*hisC10091*::Tn*phoA his*(*H* or *A*)*9556*::MudP22/pJS28 plasmid for P22 tail carries Amp^R; alias TT15089	S. Maloy
MST2003	DEL(*his-cob*)*907* DEL(*phoN*)*65*; alias TT14543	S. Maloy
MST997	*hsdL6 hsdSA29 ilv-452 metA22 trpC2 metE551 xyl-404 fla-66 rpsL120* H1-b H2-e,n,x (Fels2$^-$) nml *galE550*; alias TR5878	S. Maloy
MST3848	LT2 *pir*$^+$/pRL27e	S. Maloy
MST4208	*proBA47*/F'128 (*pro*$^+$ *lac*$^+$) *zzf-3834*::Tn*10dTet* (DEL20 DEL25)	S. Maloy
MST3271	*leu414* (Am) (Fels2$^-$) *supE dnaQ*::Tn*10*	S. Maloy
MST2817	*hsdL6 hsdSA29 hsdSB121 galE856 xyl-404 leu-3121 metA22 metE551 trpC2 ilv-452 rpsL120*	S. Maloy
MST2627	*rfc-458* ; alias SL428; (SGSC# SGSC68); Rfc$^-$; P22^R Ffm^R FO^S	S. Maloy
MST5192	Nitrosoguanidine-treated MST 1757. Mutated in phage P22 maturation.	
MST5193	Nitrosoguanidine-treated MST 1757. Mutated in phage P22 maturation. Deficient in P22 transduction.	
MST5194	Nitrosoguanidine-treated MST 1757. Mutated in phage P22 maturation.	

[a] This study, unless otherwise noted.

Appendix 1: List of Strains, Continued.

Strains	Genotype	Reference[a]
MST5195	Nitrosoguanidine-treated MST 1757. Mutated in phage P22 maturation.	
MST5196	Nitrosoguanidine-treated MST 1757. Mutated in phage P22 maturation.	
MST 4190	*leuBCD485 trp*::[Spc^R T7 gene 1 (T7 RNA polymerase) *lacp lacI*]	C. Miller

[a] This study, unless otherwise noted.

Appendix 2: Mechanism of superinfection exclusion by the SieA protein

Abstract

Although the SieA protein that is expressed by a P22 lysogen has been shown to be responsible for excluding superinfecting DNA, the mechanism of how SieA prevents superinfection is not known. The SieA protein has been shown to localize to the cytoplasmic membrane of *Salmonella* Typhimurium. Another protein that associates with the cytoplasmic membrane of *Salmonella* Typhimurium is the gp16 pilot protein that is co-ejected with the P22 DNA. Together with gp7 and gp20, gp16 is responsible for targeting, anchoring, and transporting the P22 DNA across the cytoplasmic membrane of *Salmonella* Typhimurium. Since both gp16 and SieA are closely associated with the cytoplasmic membrane, we hypothesized that SieA may interact with gp16 to prevent the superinfecting DNA from infecting the P22 lysogen. Protein-protein interaction between gp16 and SieA was studied using pull down and co-immunoprecipitation assays. The possibility that SieA might interact with the superinfecting P22 DNA was investigated by performing gel retardation assays. 6xHis-gp16 was found to co-elute with GST-SieA and to co-immunoprecipitate with Nus-SieA. Nus-SieA also co-eluted with 6xHis-gp16 in the reverse pull down assay. The protein-protein interaction between 6xHis-gp16 and GST-SieA or Nus-SieA provides convincing evidence that gp16 associates with SieA. The gel retardation assays suggest that SieA does not bind DNA. Together, these results suggest that SieA excludes superinfecting DNA by directly associating and modifying the function of gp16.

Introduction

P22 lysogens express the c2, the SieB and the SieA proteins to prevent superinfection by related phage (101). Like the λ cI protein, the P22 c2 protein also prevents growth of superinfecting P22 and related phages with similar *immC* regions by repressing lytic gene transcription (130). The SieB protein inhibits the lytic development of certain superinfecting phages by aborting macromolecular synthesis; however, P22 expresses the Esc protein which bypasses the effect of the SieB protein (174, 175). The SieA protein excludes superinfecting DNA at an earlier stage and the exclusion is stronger than SieB (40, 173).

The *sieA* gene has been sequenced (66). Although the SieA protein seems to localize at the cytoplasmic membrane, the function of the SieA protein is still not known. The superinfecting DNA in *sieA*$^+$ lysogens remains trapped in the periplasmic space where it is rapidly degraded by endogenous nucleases (173). Based on these experiments, it has been proposed that SieA may inactivate the DNA transport apparatus by interaction with an unknown component at the cytoplasmic membrane (101). The SieA exclusion may be due to inactivation of one of the P22 pilot proteins. Alternatively, superinfection exclusion may inactivate a host component which interacts with one or more of these P22 proteins (101).

Another protein that associates with the cytoplasmic membrane of *Salmonella* Typhimurium is the gp16 pilot protein that is co-ejected with the P22 DNA (70). Together with gp7 and gp20, gp16 is responsible for targeting, anchoring, and transporting the P22 DNA across the cytoplasmic membrane of *Salmonella* Typhimurium (detailed in Chapter 2). Since both gp16 and SieA are closely

associated with the cytoplasmic membrane, we hypothesized that SieA interacts with gp16 to prevent the superinfecting DNA from infecting the P22 lysogen. Protein-protein interaction between gp16 and SieA was studied using pull down and co-immunoprecipitation assays. The possibility that SieA might interact with the superinfecting P22 DNA was investigated by performing gel retardation assays.

Materials and Methods

Plasmids and strains. The pET46 Enterokinase/Ligation-Independent Cloning (Ek/LIC) vector, GST adaptor, Nus adaptor, NovaBlue cells, and the BL21 *E. coli* expression host cell were purchased from Novagen. The plasmids with the appropriate inserts were maintained in DH5α *E. coli* cells.

Chemicals. 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), isopropyl-β-D-thiogalactoside (IPTG), lysozyme, and Triton X-100 were purchased from Fisher Scientific. Rabbit anti-6xHis and mouse anti-GST antibodies were purchased from Santa Cruz Biotechnology. Goat anti-rabbit and goat anti-mouse antibodies were purchased from Jackson ImmunoResearch. His-bind affinity column purification kit, mouse anti-Nus antibody, protein G/A agarose, and benzonase were purchased from Novagen. The BCA Protein Assay kit used to determine protein concentration and the GST resin were obtained from Pierce Biotechnology, Inc.

Vector Construction. DNA sequences of genes coding for the P22 pilot protein gp16 and for the SieA protein were obtained from genbank. The P22 DNA template was extracted from concentrated P22 lysate as previously described (111). Forward and reverse primer sequences used to amplify the *sieA* gene were: forward

(5' to 3') GACGACGACAAGATGTCTGATTCTATGAGTTATGCTGTGCTA; reverse (5' to 3') GAGGAGAAGCCCGGTTTATTTTATAGTTTTTATGAATCCTTTATT. Forward and reverse primer sequences used to amplify *gene 16* were: forward GACGACGACAAGATGAAAGTTACCGCTAATGGCAAGACATTC; reverse GAGGAGAAGCCCGGTCTACTGCCGGGTAGCTTCGTTAGCTAAAAG.

The amplified gene sequences were cloned into the pET46 Ek/LIC expression vector with the GST or Nus adaptor. The presence of the appropriate inserts was confirmed by PCR, restriction digests and DNA sequencing. Expression vectors with the correct inserts were electroporated into the expression host cell line BL21 from Novagen, and then plated on LB agar plates with 100 µg/ml Ampicillin.

Protein Expression and Purification. BL21 cells with each plasmid clone were inoculated into 100 ml of LB broth with 100 µg/ml Ampicillin and incubated in a 37^{o}C shaker until mid-exponential growth. IPTG was added to a final concentration of 1 mM to induce protein expression and the cultures reincubated for an additional 3 hr in a 30^{o}C shaker. The cells were then centrifuged at 3842 x g for 30 min at 4^{o}C. The supernatant was removed and the pellet resuspended in 4 ml of lysis buffer (50 mM NaH_2PO_4, pH 8, 300 mM NaCl, 10 mM Imidazole, 10 mM CHAPS). Lysozyme was added to 0.5 mg/ml and 100 units of benzonase was also added to reduce viscosity of the lysate. The lysate was incubated in ice for 30 min with occasional swirling and then subjected 3 times to sonication using the Fisher Scientific ultrasonic dismembrator Model 100. The sonicated lysate was centrifuged at 11000 x g for 30 min at 4^{o}C. The supernatant was added to a column containing 200 µl bed volume of nickel-charged His-bind resin (Novagen) and samples eluted at a flowrate of ca. 1 ml/min. The resin was washed three times with 1X binding buffer (50 mM NaH_2PO_4,

pH 8, 300 mM NaCl, 20 mM Imidazole, 10 mM CHAPS) and washed twice with 1X wash buffer (50 mM NaH_2PO_4, pH 8, 300 mM NaCl, 60 mM Imidazole, 10 mM CHAPS) before the 6xHis-tagged ejection protein was eluted three times with 200 µl each of 1X elution buffer (50 mM NaH_2PO_4, pH 8, 300 mM NaCl, 1 M Imidazole, 10 mM CHAPS). The 1 M imidazole was removed from the eluted ejection protein by using a centricon filter with a molecular weight cut off of 5 kDa and by washing the ejection protein with 20 mM Tris buffer, pH 8, 100 mM NaCl and 0.1% Triton X-100. Protein concentration was determined using the BCA Protein Assay kit from Pierce. Alternatively, GST-tagged proteins were purified using an affinity column packed with the GST resin. 1X phosphate buffered saline (PBS) was used as the wash buffer for the GST column and 10 mM reduced Glutathione was used to elute the GST-tagged proteins. The Nus-tagged SieA protein was purified using an antibody against the Nus-tag. ProteinG/A agarose was used to bind and pellet the Nus-tag antibody bound to Nus-SieA.

Co-elution and co-immunoprecipitation. 6xHis-tagged gp16 and GST-tagged or Nus-tagged SieA were co-expressed in the same BL21 expression host cell. Nickel-charged His-bind resin (200 µl bed volume) was used to purify 6xHis-tagged gp16, GST resin (200 µl bed volume) was used to purify GST-tagged, and anti-Nus antibody was used to immunoprecipitate Nus-SieA. The co-eluted protein was detected using rabbit anti-6xHis, mouse anti-GST or mouse anti-Nus. To immunoprecipitate Nus-SieA, 25 µg of the anti-Nus antibody was added to the cleared lysate and incubated for 2 hr with gentle rocking at 4^{o}C. This was followed by the addition of 40 µg of protein G/A agarose and rocking was continued for another hour at 4^{o}C before the samples were centrifuged at top speed for 5 min in a clinical

centrifuge to pellet the protein G/A agarose. After extensive washing with a buffer consisting of 20 mM Tris, 100 mM NaCl, 0.1% Triton X-100, the protein G/A agarose was boiled in SDS loading buffer for 5 min at 99°C and the samples were separated on a 10% SDS polyacrylamide gel. The proteins were transferred on a polyvinylidene fluoride (PVDF) membrane and the co-immunoprecipitated protein was detected using the rabbit anti-6xHis antibody.

Western Blot. Primary antibodies were used at 1:1500 dilution. The goat anti-rabbit and goat anti-mouse secondary antibodies conjugated with horseradish peroxidase were used at 1:10,000 and 1:5,000 dilution, respectively.

Labeling of DNA with ^{32}P. The *bla* DNA was PCR-amplified from the pUC19 vector by using the following forward and reverse primers: forward: 5'-GACGACGACAAGATGAGTATTCAACATTTCCGTGTCGCCCTTATTCCC-3'; reverse: 5' GAGGAGAAGCCCGGTTTACCAATGCTTAATCAGTGAGGCACCTATCTC-3'. The *gene20* DNA was PCR-amplified from purified phage P22 DNA by using the following forward and reverse primers: forward: 5'-GACGACGACAAGATGGCTACGTGGCAGCAGGGCATTAATTCAGGT; reverse: 5'-GAGGAGAAGCCCGGTTTATTCCACCGTGAATTTAATGCCAGATTT. Both PCR products were 5' end labeled using T4 polynucleotide kinase (New England Biolabs) and 20 µCi of [γ-^{32}P]ATP (154).

Gel Retardation Assays. Purified 6xHis-SieA (100 nM final concentration) was incubated with a final concentration of 1 nM of ^{32}P-*bla* DNA for 1 hr at 4°C. All reaction mixtures were in 20 mM Tris pH 8, 100 mM NaCl in a total volume of 36 µl.

Reactions were mixed with a sucrose dye solution (153) and separated on a 5% native polyacrylamide gel. Vacuum-dried gels were exposed to a phosphorimager screen and analyzed using Molecular Dynamics ImageQuant v. 5.2 software.

Results

Both gp16 and SieA have been shown to closely associate with the cytoplasmic membrane of *Salmonella*. The interaction between these two proteins could potentially explain the mechanism of superinfection exclusion performed by the SieA protein of a P22 lysogen. We hypothesized that SieA interacts with gp16 to prevent the superinfecting DNA from infecting the P22 lysogen. Protein-protein interaction between gp16 and SieA was studied using pull down and co-immunoprecipitation assays. 6xHis-gp16 was found to co-elute with GST-SieA (Figure 6.1A) and to co-immunoprecipitate with Nus-SieA (Figure 6.1C). Nus-SieA also co-eluted with 6xHis-gp16 in the reverse pull down assay (Figure 6.1B). The protein-protein interaction between 6xHis-gp16 and GST-SieA or Nus-SieA provides convincing evidence that gp16 associates with SieA.

The SieA protein might interact directly with the superinfecting DNA to prevent it from entering the P22 lysogen. The possibility that SieA might interact with the superinfecting P22 DNA was investigated by performing gel retardation assays. The gel retardation assay in Figure 6.2A shows that 6xHis-SieA does not bind ^{32}P-labeled *gene 20* DNA which was PCR-amplified from the P22 genome. To rule out the possibility that SieA might bind P22 DNA nonspecifically, binding of SieA to the ^{32}P-labeled *bla* DNA was investigated. The result of the gel retardation assay depicted in

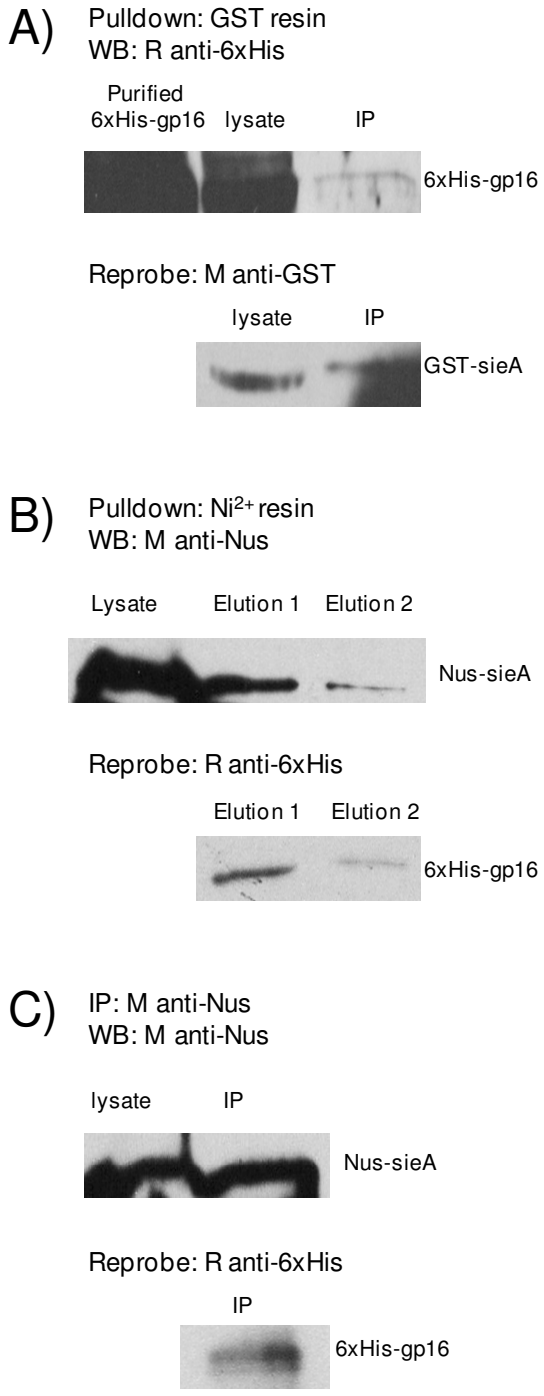


Figure 6.1: *gp16 interacts with the SieA protein.* (A) 6xHis-gp16 coelutes with GST-SieA. (B) Nus-SieA coelutes with 6xHis-gp16. (C) 6xHis-gp16 co-immunoprecipitates with Nus-SieA.

Figure 6.2B shows that 6xHis-SieA does not bind DNA nonspecifically. This was expected since SieA is known to also exclude superinfecting DNA coming from transducing particles carrying bacterial DNA. 6xHis-gp7, 6xHis-gp16, and 6xHis-gp20 were used as positive controls since they were previously shown to bind DNA nonspecifically.

In summary, these results suggest that the P22 SieA protein forms a complex with gp16. SieA also does not associate with any DNA which suggests that the way SieA excludes superinfecting DNA is not by interacting with the DNA directly but by associating and modifying the function of gp16.

Discussion

The mechanism of superinfection exclusion was shown to involve the interaction of SieA and gp16. Binding of the SieA protein to gp16 and not to DNA indicated that the mechanism of excluding superinfecting DNA is by preventing gp16 in initiating DNA transport and not by directly blocking the incoming phage DNA. The fact that P22 *sieA*$^{+}$ lysogens can exclude superinfecting phage L DNA accompanied by pilot proteins that are homologous to the P22 pilot proteins (63, 76, 158, 185) provides further evidence that the SieA protein interacts directly with one or more of the pilot proteins to exert its influence. Moreover, since a P22 *sieA*$^{+}$ lysogen cannot exclude superinfecting DNA from KB1 or 9NA (16), this supports the idea that the SieA protein does not directly bind the superinfecting DNA to prevent it from entering the host cytoplasm.

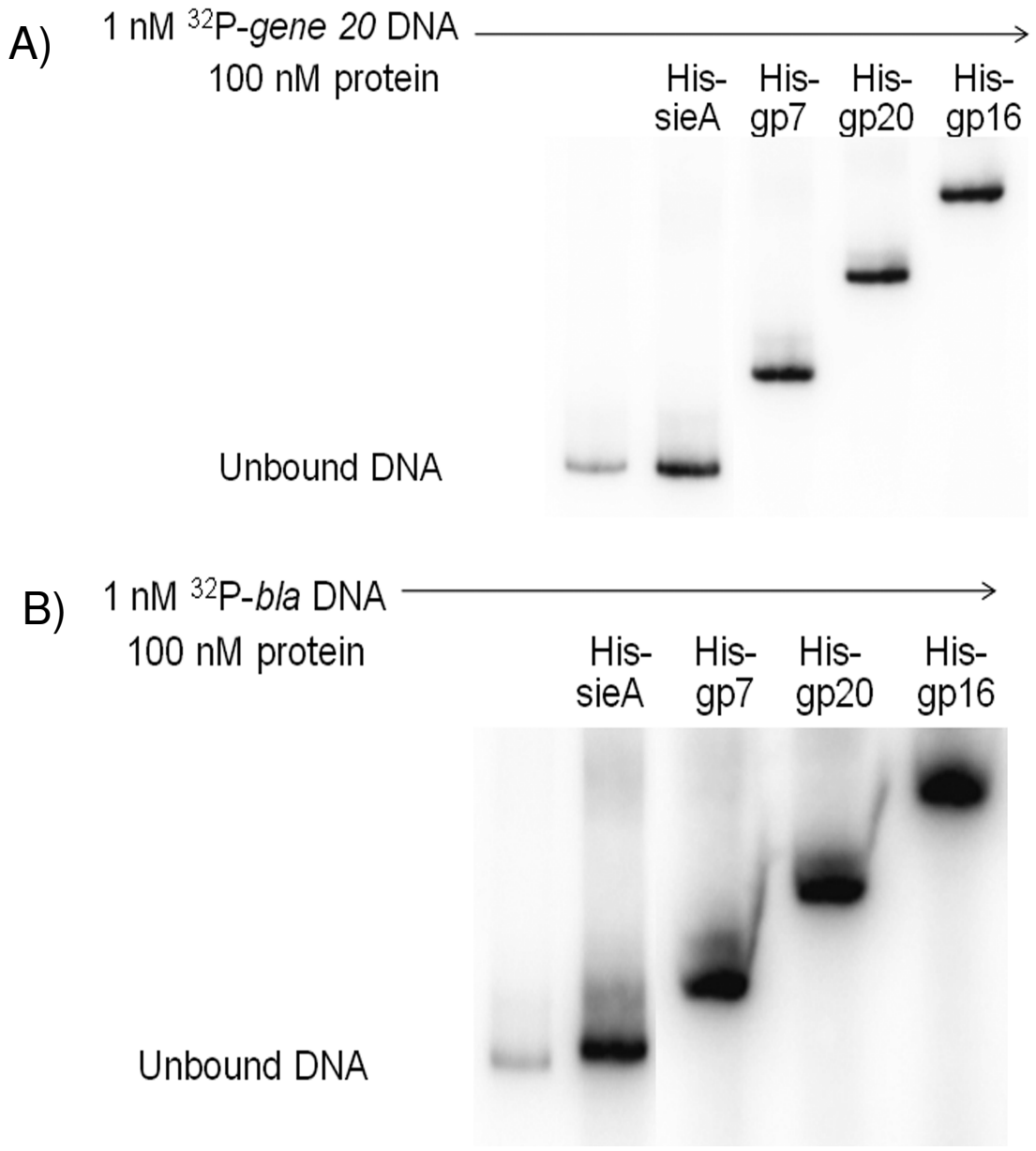


Figure 6.2: *SieA does not bind DNA.* (A) 6xHis-SieA does not bind ^{32}P-labeled *gene 20* DNA. 6xHis-gp7, 6xHis-gp16 and 6xHis-gp20 were used as positive controls since they have been shown to bind DNA nonspecifically. (B) 6xHis-SieA does not bind ^{32}P-labeled *bla* DNA.

The possibility that the SieA-mediated exclusion requires interaction of the SieA protein with gp7 and gp20 still needs to be investigated. In addition, the existence of a host membrane protein that might be involved in the SieA-mediated

exclusion of incoming phage DNA has not been ruled out. Further genetic and biochemical manipulations have to be performed to identify host factors serving as liaisons for both the SieA and the gp16 pilot protein in the mechanism of superinfection exclusion.

Mechanism of P22 superinfection exclusion by SieA. The investigation of the nature of interaction between SieA, gp16, and DNA revealed that SieA interacts with gp16 but does not bind DNA. Based on these results, one model of how SieA excludes superinfecting DNA is by inhibiting the gp16 protein from reaching the surface of the cytoplasmic membrane and initiating channel formation (Figure 6.3a). The potential interaction of SieA to gp7 and gp20 affords SieA another opportunity to prevent phage DNA uptake (Figure 6.3b). Failing to inhibit anchoring of the gp16 protein to the cytoplasmic membrane, SieA could still alter the conformation of the transmembrane gp16 protein such that it can no longer facilitate uptake of the phage DNA (Figure 6.3c). Since the existence of a host transporter protein was not completely eliminated, exerting pressure on this unidentified host protein might serve as another pathway for SieA-mediated exclusion (Figure 6.3d). The availability of all these options for superinfection exclusion has yet to be determined. Thus far, what has been established is that SieA does not directly bind DNA which would imply that superinfection exclusion does not involve directly interacting with the incoming phage DNA.

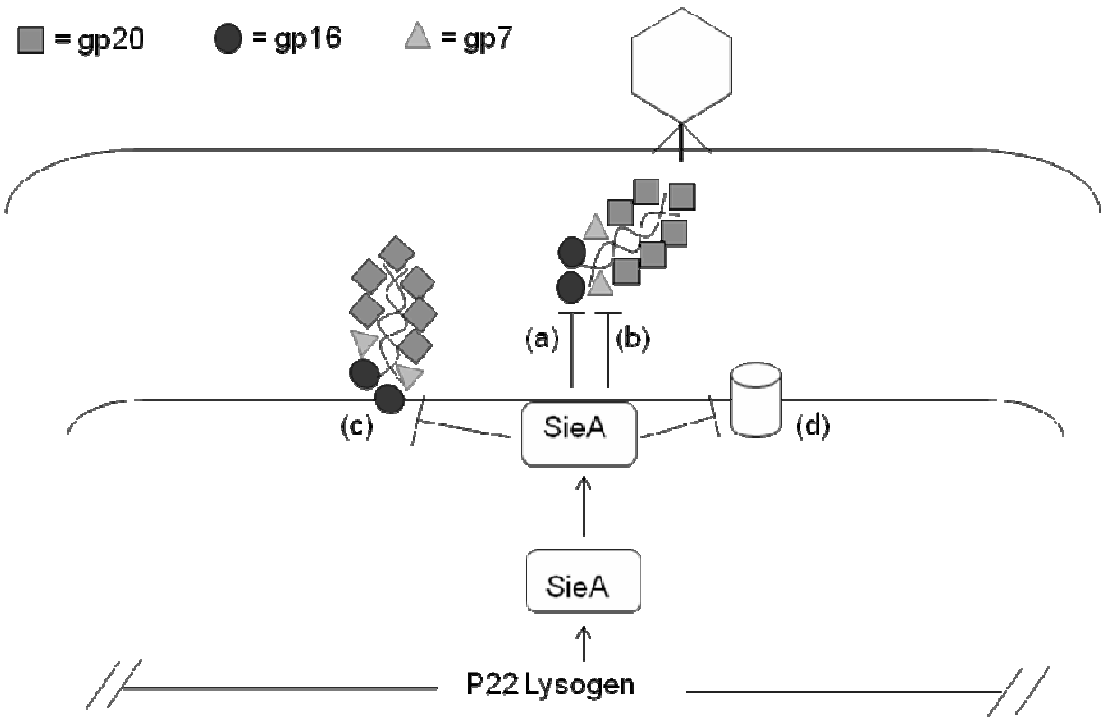


Figure 6.3. *Model of superinfection exclusion.*

Future experiments:

1) Does the overexpression of gp16 overcome SieA-mediated superinfection exclusion? This question can be answered by transducing a P22 *sieA*$^{+}$ lysogen with transducing particles that not only have packaged a selectable marker but also contains 10 to 100-fold more gp16 pilot proteins inside the capsid. To this end, the 6XHis-gp16 gene can be overexpressed in a *Salmonella* expression host cell by IPTG induction of the pET30 expression vector carrying both the gp16 gene and the Kanamycin resistant cassette. The pET30 vector can be packaged in P22 HT105/1 *int*-201 by infecting the *Salmonella* expression host cells which are loaded with the 6XHis-gp16 pilot proteins. The P22 transducing particles will not only carry the Kanamycin selectable marker from the pET30 plasmid but will also contain more

copies of the gp16 protein than usual. To determine how much more gp16 proteins have been packaged in the transducing particles, the 6XHis-gp16 protein from transducing particles generated from IPTG-induced and uninduced expression host cells can be compared by Western blotting with rabbit anti-6XHis antibody. Equal titers of the phage lysates generated from the induced and uninduced *Salmonella* expression host cells will be used to transduce a P22 *sieA*$^{+}$ lysogen and the transduction efficiency of the phage particles carrying more copies of the gp16 protein can be directly compared to the transduction efficiency of the phage particles with a normal number of copies of the gp16 protein. Since the SieA protein was shown to interact with the gp16 protein, it is predicted that the overexpression of the gp16 protein would overcome SieA-mediated superinfection exclusion. If the overexpression of gp16 does not overcome the exclusion presented by SieA, then that would suggest that SieA may also be inhibiting gp7, gp20 or a host membrane protein. One way or another, the outcome of this venture would provide additional insights into the mechanism of superinfection exclusion by SieA and perhaps even unravel some of the mysteries surrounding the mechanism of P22 DNA transport.

2) Does SieA interact with the other pilot proteins? Similar to the procedure followed for testing the interaction between SieA and gp16, pull down or co-immunoprecipitation assays can be performed to investigate the interaction of SieA to either gp7 or gp20. It would be worthwhile exploring the use of Surface Plasmon Resonance as an alternative approach since this technique is quantitative, follows protein-protein interaction in real time, and can also provide the kinetics of the interaction between the proteins being studied.

3) Does SieA inhibit gp16 before it reaches the surface of the cytoplasmic membrane thereby preventing gp16 from anchoring to the cytoplasmic membrane or does SieA modify gp16 when it has already penetrated the cytoplasmic membrane? One way to answer this question is to perform a variation of the liposomal DNA transport assay. Liposomes will either be reconstituted in SieA alone or in gp16 and SieA such that these proteins are membrane-bound. Soluble gp16 will be added to membrane-bound SieA separately. Radiolabeled DNA transport will be assayed in the soluble gp16 vs. the membrane-bound gp16. If DNA transport occurs only in the presence of soluble gp16 and not in the membrane-bound gp16, then SieA must be inhibiting gp16 when it is already membrane-bound. Alternatively, if no DNA gets transported when soluble gp16 is added separately to the liposomes with membrane-bound SieA, then that would suggest that SieA is capable of inhibiting gp16 while it is on its way to the surface of the cytoplasmic membrane. Lastly, if no DNA gets transported at all, then that would suggest that SieA can inhibit gp16 whether it is in the soluble form or in the membrane-bound conformation. This question may also be better answered when the different conformations of gp16 (before and after membrane association) are known. The SieA protein might have a higher affinity towards one conformation over the other thereby inhibiting only one specific conformation of gp16.

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